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The Interaction of Polynuclear Hydrocarbons With Mouse-Skin Protein [1, 2, 3]

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Summary

Binding to protein was observed when a series of four C^{14}-labeled polynuclear hydrocarbons were mixed with disintegrated mouse skin. Although processes *in vivo* may occur, such interaction prevents the conclusive demonstration of the binding *in vivo* of any chemical substance when methods involving cellular disintegration are employed.—J. Nat. Cancer Inst. 22: 661–668, 1959.

The supposition that carcinogenic substances elicit their response by interaction with tissue has directed much effort toward the identification of critical tissue constituents. The extensive work of Miller and Miller (*1*, *2*), Moodie, Ried, and Wallick (*3*), and Heidelberger and Moldenhauer (*4*) has indicated an important role for tissue protein in carcinogenesis. Insofar as skin is concerned, Woodhouse (*5*, *6*) and Hadler, Darchun, and Lee (*7*) have reported that the experiments which related carcinogenesis to the binding of carcinogens or their metabolites with protein were not decisive.

The sequence of events in the experimental procedures generally used were the following: application of the compound to the skin of the live animal, isolation of the treated skin, disintegration of the skin (some investigators disintegrated the skin and/or precipitated the protein in the presence of a solvent presumably to remove unbound material), precipitation of the protein, and an exhaustive extraction of the precipitated protein with inorganic and organic solvents.

The isolation of a complex between the protein and the original compounds or their metabolites was taken to mean (*1–8*) that a combination *in vivo* had occurred between the elements of the complex. In many instances the degree of protein binding, subsequent to the application of a single dose of the chemical agent, first rose, reached a maximum, and then

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[3] An abstract of some of this work appeared in Proc. Am. Assoc. Cancer Res. 2: 304. 1958.

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diminished. Such rate data only authenticated the occurrence of a sequence of unknown events *in vivo* which stemmed from the application of the agent.

It has been reported from this laboratory that after the application of the noncarcinogenic hydrocarbons anthracene-9,10-C^{14} and 9-methylanthracene-9,10-C^{14} to the skin of live mice (*7*), the degree of protein binding started from a maximum, fell, and never rose above the initial value. Rate data alone were thus insufficient to establish a sequence of unknown processes *in vivo* for the tricyclic hydrocarbons. Subsequently, Gutman and Peters (*9*) reported that the binding of *N*-2-fluoren-9-C^{14}-ylacetamide and of 2-fluorenamine-9-C^{14} with protein of rat skin was induced during the homogenization of the tissue and/or during the precipitation of the protein. Such binding was extensive with the free amine. The binding *in vitro* of the kind observed by Gutman and Peters became a distinct possibility in the anthracene and 9-methylanthracene experiments (*7*).

Two questions emerged from these considerations: 1) Did a sequence of unknown events *in vivo* stem from the application *in vivo* of the two tricyclic hydrocarbons? 2) In the experiments *in vivo* with the tetracyclic hydrocarbon (*8*) was protein binding included among the sequence of unknown events *in vivo*? Experiments related to these problems were undertaken with four C^{14}-labeled polynuclear hydrocarbons.

Experiments

In vivo.—The hydrocarbons, 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} (*10*), benz[*a*]anthracene-7,12-C^{14}, anthracene-9,10-C^{14}, and 9-methylanthracene-9,10-C^{14} (*11*) were all prepared from the same lot of phthalic anhydride-7-C^{14}; they had a specific activity of 2.77 mc. per mmole and were purified as described (*10*, *11*). The mice were painted and the protein isolated and counted as previously described (*8*). A Teflon pestle rather than a glass pestle was used for homogenization.

Induced binding.—The labeled hydrocarbon was deposited from an acetone solution on the lower sides of a homogenizer tube, while the inclined tube was slowly rotated in a warm-water bath. Either the gross supernatant fraction (*8*), or the gross particulate fraction, or the appropriate precipitated protein fraction (*8*) was added to the tube. The tube was surrounded by an ice-water bath and 20 strokes were made with the Teflon pestle in the case of 7,12-dimethylbenz[*a*]anthracene and benz[*a*]-anthracene and 25 strokes in the case of anthracene and 9-methylanthracene. After the mixing, the denatured purified protein was isolated by completing the appropriate operations (*8*).

Degradation.—The previously described procedure (*12*), which was used to degrade the protein complex derived from mouse skin and dibenz[*a*,*h*]anthracene-7,14-C^{14}, was utilized. The pepsin concentration was 0.1 percent and the incubations were at 37° C. for about 17 hours. The saponifications were carried out for about 17 hours under reflux with 5 N NaOH. The degradation procedure gave the following fractions: (*a*) an insoluble residue resistant to pepsin; (*b*) a fraction extracted by

ethyl acetate from the pepsin digest (the first extract of table 1); (*c*) a pepsin digest which had been extracted with ethyl acetate; (*d*) neutrals extracted by benzene from the alkaline solution (the second extract of table 1); (*e*) free acidic material (acids plus phenols) extracted by ethyl acetate from the alkaline solution after acidification.

Results and Discussion

It was possible to obtain protein binding *in vitro* by the admixture of equivalent quantities of the 4 polynuclear hydrocarbons with the gross supernatant and gross particulate fractions (*8*) of previously disintegrated mouse skin (table 2). The total radioactivity in the homogenates *in vitro* (table 2) indicated that the dispersion *in vitro* of the labeled material was neither complete nor reproducible. Consequently the total radioactivity in the homogenates (the particulate and supernatant fractions in the experiments *in vitro* and the whole homogenate in the experiments *in vivo*) was used as a practical basis when comparing the binding efficiency of the various experiments. It was more difficult to obtain a dispersion *in vitro* of the 2 tricyclic hydrocarbons than of the 2 tetracyclic hydrocarbons. The supernatant protein was more efficient than the particulate protein as a binding receptor. When precipitated skin protein was mixed with 7,12-dimethylbenz[*a*]anthracene similar binding data *in vitro* were obtained. The binding, induced *in vitro*, was more facile with anthracene and 9-methylanthracene than with the 2 tetracyclic hydrocarbons (table 2).

Maximum radioactivity of the precipitated protein was attained subsequent to a decided loss of radioactivity from the painted area—in the rate experiments *in vivo* with 7,12-dimethylbenz[*a*]anthracene (*8*), as noted previously, and with benz[*a*]anthracene (table 3). Thus a sequence of unknown processes *in vivo* followed the application of each of the 2 tetracyclic hydrocarbons. The magnitude of the binding induced *in vitro* with these tetracyclic hydrocarbons was insufficient to challenge seriously this conclusion.

The binding induced *in vitro* with both tricyclic hydrocarbons (table 2) was extensive enough to dispute the occurrence of a sequence of unknown events *in vivo* in the previously published experiments *in vivo* (*7*). This result and the rate studies *in vivo* (*7*) required additional data concerning the nature of the labeled precipitated protein isolated from the experiments *in vivo* and *in vitro*.

All the proteins were further examined by the methods devised to identify protein-bound metabolites of hydrocarbons, *i.e.*, pepsin digestion, extraction with ethyl acetate, and saponification (*12*). The 2 classes of labeled protein, one isolated after the application *in vivo* of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} and the other isolated after binding was produced *in vitro* yielded radioactivity in the washed digest. Thus, in the 2 instances, there was radioactivity which remained associated with water-soluble polypeptides. It was significant that the proportions of protein-bound radioactivity recovered in the washed digests associated

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TABLE 1.—*Degradation of labeled protein*

Protein sample:*	1	2	3	4	5	6	7	8	9	10	11
Particulate protein: Percent radioactivity in residue	33	38	63	59	23	49	22	50	47	24	59
First extract †	4	3	‡	2	‡	‡	3	2	3	3	7
Washed digest	72	56	26	20	59	41	40	45	16	53	15
Second extract §	‡	3	4	2	‡	‡	‡	5	3	1	4
Acids and phenols	30	9	‡	‡	15	‡	11	10	8	11	4
Supernatant protein: Percent radioactivity in first extract †	‡	12	11	9	‡	‡	6	7	12	6	17
Washed digest	62	68	14	27	57	34	80	80	43	53	44
Second extract §	‡	5	3	‡	‡	‡	‡	14	6	‡	11
Acids and phenols	23	14	‡	‡	24	‡	19	19	14	19	11

* The protein samples were identified by the same numbers which identified the experimental conditions in table 2. Duplicate protein samples were combined previous to digestion with pepsin.

† The ethyl acetate extract following digestion with pepsin.

‡ The amount of radioactivity was too low for measurement.

§ The benzene extract following alkaline hydrolysis.

TABLE 2.—*The labeling of precipitated protein*

TABLE 2.—*The labeling of precipitated protein*

Experimental conditions*	1	2	3	4	5	6	7	8	9	10	11
Whole homogenate activity (c/m) †	373, 000	316, 000 268, 000			273, 000		371, 000	67, 500 80, 000		1, 380, 000	
Particulate protein:											
Homogenate activity (c/m)	‡	‡	300, 000 372, 000	‡	‡	263, 000 284, 000	‡	‡	42, 500 23, 500	‡	73, 800 68, 200
Specific activity (c/m/mg.)	410	83 96	41 34	100 90	410	40 59	600	180 130	270 95	1600	200 360
Supernatant protein:											
Homogenate activity (c/m)	‡	‡	68, 400 133, 000	‡	‡	264, 000 35, 000	‡	‡	51, 500 15, 700	‡	64, 100 4600
Specific activity (c/m/mg.)	610	290 300	220 390	90 260	750	223 107	1500	400 560	860 540	2300	1300 160

*The protein samples 1 to 11, except 5, were obtained from the pooled skins of 8 mice, and the results were tabulated on a basis of 4 mice. Protein samples, 5, were obtained from the pooled skins of 4 mice, and the results tabulated on the basis of 4 mice. The duplicate values reported are from separate experiments, each with 8 mice. Other pertinent data follow:

1) In this experiment *in vivo* the applied dose of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} was 150 μg. (1,860,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 110 μg. (1,365,000 c/m). The mice were killed 1 day after the application of the hydrocarbon.

2) In this experiment *in vivo* the applied dose of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} was 10 μg. (124,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 7.3 μg. (91,000 c/m). The mice were killed 1 hour after the application of the hydrocarbon.

3) This was an *induced binding* experiment with 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14}. The homogenizer tube contained 7.3 μg. (91,000 c/m) of hydrocarbon per appropriate skin fraction derived from 2.54 cm.^{2} of skin. The pooled tissue fractions of 8 mice were in 1 homogenizer tube.

4) This was an *induced binding* experiment with 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14}. The homogenizer tube contained 7.3 μg. (91,000 c/m) of hydrocarbon per appropriate precipitated protein fraction derived from 2.5 cm.^{2} of skin. The pooled tissue fractions of 8 mice were in 1 homogenizer tube.

5) In this experiment *in vivo* the applied dose of benz[*a*]anthracene-7,12-C^{14} was 133 μg. (1,860,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 98 μg. (1,365,000 c/m). The mice were killed 16 hours after the application of the hydrocarbon.

6) This was an *induced binding* experiment with benz[*a*]anthracene-7,12-C^{14}. The homogenizer tube contained 8.9 μg. (91,000 c/m) of hydrocarbon per appropriate skin fraction derived from 2.5 cm.^{2} of skin. The pooled tissue fractions of 8 mice were in 1 homogenizer tube.

7) In this experiment *in vivo* the applied dose of anthracene-9,10-C^{14} was 104 μg. (1,860,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 76 μg. (1,365,000 c/m). The mice were killed 30 minutes after the application of the hydrocarbon.

8) In this experiment *in vivo* the applied dose of anthracene-9,10-C^{14} was 7 μg. (124,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 5.1 μg. (91,000 c/m). The mice were killed 20 minutes after the application of the hydrocarbon.

9) This was an *induced binding* experiment with anthracene-9,10-C^{14}. The homogenizer tube contained 5.1 μg. (91,000 c/m) of hydrocarbon per appropriate skin fraction derived from 2.5 cm.^{2} of skin. The pooled tissue fractions of 8 mice were in 1 homogenizer tube.

10) In this experiment *in vivo* the applied dose of 9-methylanthracene-9,10-C^{14} was 113 μg. (1,860,000 c/m) per 3.5 cm.^{2} of mouse skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 83 μg. (1,365,000 c/m). The mice were killed 30 minutes after the application of the hydrocarbon.

11) This was an *induced binding* experiment with 9-methylanthracene-9,10-C^{14}. The homogenizer tube contained 5.5 μg. (91,000 c/m) of hydrocarbon per appropriate skin fraction derived from 2.5 cm.^{2} of skin. The pooled tissue fractions of 8 mice were in 1 homogenizer tube.

†c/m = count per minute.

‡Not measured.

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with the experiments *in vivo* were markedly higher (table 1). Furthermore, the saponification of this digest gave rise to a gross acidic fraction (acids plus phenols). No such gross acidic fraction was recovered after the saponification of the digest derived from the other class of protein which had been labeled by the procedure *in vitro*. These data are further independent evidence for the occurrence of a process *in vivo* with 7,12-dimethylbenz[*a*]anthracene. The results and conclusions obtained with benz[*a*]anthracene (table 1) were similar.

Comparable protein-degradation data were obtained for anthracene. Both classes of labeled protein yielded radioactivity in their respective washed digests. Again the proportions of protein-bound radioactivity recovered in the washed digests associated with the experiments *in vivo* were significantly higher. The gross acidic fraction (acids plus phenols) was recovered, however, following the application of the hydrocarbon both *in vivo* and *in vitro*. The other tricyclic compound, 9-methylanthracene, which was less extensively studied, gave results consistent with those of anthracene. Thus, only the recovery of radioactivity in the various washed digests established that some metabolic process *in vivo* was extant following the application *in vivo* of both anthracene and 9-methylanthracene.

Analogous binding *in vivo* of another polynuclear hydrocarbon has been reported by others. Binding of dibenz[*a*,*h*]anthracene-7,14-C^{14} was induced with the protein of the submaxillary gland of mice (*13*) and with the protein of the supernatant fraction of mouse skin (*14*). The specific activity of the supernatant skin protein labeled *in vitro* was 45 percent of the maximum value of the experiments *in vivo*. The binding, *in vitro*, with the protein of the particulate fraction of mouse skin was not significant (*14*). The higher specific activity of the supernatant skin protein (*14*) agreed with the observations of the present study. The variable results observed with the submaxillary gland of mice (*13*) were due, most likely, to the difficulty of dispersion as found in this study.

Conclusions

The protein binding induced *in vitro* with anthracene, 9-methylanthracene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, dibenz[*a*,*h*]-anthracene (*13*, *14*), *N*-2-fluorenylacetamide and 2-fluorenamine (*9*) does suggest that metabolites as well as the original chemical agent which were *not bound in the intact tissue or cell* may have become bound by a process which occurred as a consequence of the disintegration of the tissue or cell. Such interaction might occur at any time after the application of the agent *in vivo*. By the experimental method used herein, the isolation of a chemical agent or its metabolite bound to precipitated protein (*1–8*) is not conclusive evidence that the binding process occurred *in vivo*. Much of the rate data in the literature on binding *in vivo* which have related protein binding to carcinogenesis do not necessarily relate protein binding *in vivo* with carcinogenic potentiality. Other experimental approaches are needed to determine the role of tissue protein in carcinogenesis.

TABLE 3.—*Incorporation of radioactivity by mouse skin following the application* in vivo *of benz[a]anthracene-7,12-C^{14}* *

Time after application (*hrs.*):	0. 5	16	24
Whole homogenate (c/m)	1, 690, 000 2, 220, 000	273, 000 362, 000	103, 000 124, 000
Particulate protein (c/m/mg.)	250 160	410 580	335 460
Supernatant protein (c/m/mg.)	140 310	750 690	580 700

*Each determination was made on the pooled tissues of 4 mice; the applied dose was 133 μg. (1,860,000 c/m) of hydrocarbon over 3.5 cm.^{2} of skin; the dose which corresponded to the 2.5 cm.^{2} of skin excised was 98 μg. (1,365,000 c/m).

The attempted demonstration of the binding *in vivo* of any chemical substance or its metabolite to cellular constituents is inconclusive when the above methods of cellular disintegration and protein precipitation are utilized.

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Transplantable Chloromyeloid Leukemia in Sprague-Dawley Rats Following Injection of Actinium-227 [1, 2]

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Summary

Chloroleukemia in the Sprague-Dawley rat presumably initiated by actinium-227 (actinium-equilibrium mixture) has been successfully transplanted in suckling rats over a period of 4 years. The morphologic and anatomic characteristics are described and found to be similar to those induced by methylcholanthrene. The successful transfer of the chloroleukemia in suckling rats was accomplished with an intraperitoneal injection of 0.1 to 0.2 ml. of leukemic whole blood without any detectable changes, after 4 years of transplantation, in either morphologic and anatomic properties or the lifespan of the leukemic animal. The chloroleukemia was also successfully transplanted in suckling rats by injection of tissue homogenates obtained from leukemic rats. The longevity of the animals injected with successful transplants appears to be a function of the number of myelocytes injected. This fact plus our inability to obtain successful transplants of the chloroleukemia with cell-free extracts leads us to conclude that leukemogenesis occurs most likely through a process of colonization.—**J. Nat. Cancer Inst. 22: 669–683, 1959.**

Myelogenous leukemia developed (1954) in two noninbred stock Sprague-Dawley rats of several hundred that had been given single intravenous injections of 0.17 μc and 0.44 μc of actinium-227 [actinium-equilibrium mixture (AEM)] per kilogram of body weight to determine the chronic effect on the hematopoietic system. The leukemia was identified by peripheral-blood studies, in one animal at 9 months of age and in the other at 20 months of age, post injection of the radioisotope.

No incidence of spontaneous myelogenous leukemia has been reported at the Sprague-Dawley breeding laboratories,[4] nor at the Mound Laboratory [5] in diverse experimental studies involving many thousands of

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[2] This work was supported in part by the Charles F. Kettering Foundation, Yellow Springs, Ohio; the Montgomery County, Ohio, Society for Cancer Control; and the Ella Lowe Gunckle Fund, Dayton, Ohio.

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[4] Personal communication, Sprague-Dawley Farms, Inc., Madison, Wis.

[5] The Mound Laboratory is operated by the Monsanto Chemical Company, Miamisburg, Ohio, under U.S. Government Contract Number AT 33-1-Gen-53. The Biology Division of the Mound Laboratory was directed by Dr. David S. Anthony, and the animal research was carried out under the guidance of Dr. Robert K. Davis. Since the cessation of the activities of the Biology Division in 1955, the Mound strain of leukemic rats has been maintained at the Miami Valley Hospital by the Department of Research under the direction of Dr. Robert E. Zipf.

Sprague-Dawley rats. It is, therefore, assumed that these leukemias were radiation-induced. The carcinogenic action of radioisotopes is well documented (*1*).

This leukemia was found to be transplantable and has been carried in young rats by injection of blood from leukemic host rats. There were 7 subtransfers at the Mound Laboratory and 22 subtransfers at the Miami Valley Hospital, Department of Research.

This report is a documentation of the chloromyeloid leukemia in rats from results obtained at the Miami Valley Hospital, and is a reaffirmation of some of the early initial observations made at the Mound Laboratory (*2*), which have been extended by further work.

Experiments

Materials and Methods

Noninbred (stock) rats.—In the initial studies, the animals that received actinium-227 were stock Sprague-Dawley rats. Subsequently, sucklings of randombred Sprague-Dawley rats were used as a host for transplantation of the leukemic cells. No attempt has been made to develop inbred strains from the stock animals.

Preparations and procedures.—Transplants into suckling rats were made with either whole blood, ascitic fluid, or tissue homogenates from leukemic rats. Tumor tissue, liver, spleen, muscle, or kidney tissue was homogenized and aliquots injected intraperitoneally into the host.

The diagnosis of chloroleukemia was made from evidence of leukemic cells in the peripheral-blood smears (Wright's stain), supravital preparations, white blood cell counts, or a combination of these procedures (*3*). A complete autopsy, including examination of the brain, was performed on each animal. Histopathologic studies were made on formalin-fixed paraffin-embedded sections stained with hematoxylin and eosin on all dead or killed animals.

Suckling rats less than 7 days of age were injected intraperitoneally with: 1) 0.1 or 0.2 ml. of leukemic whole blood obtained by cardiac puncture from ether-anesthetized leukemic rats; 2) 0.2 ml. ascitic fluid obtained by paracentesis (either with or without washing of the peritoneal cavity with saline); or, 3) 0.2 ml. of 50 percent by volume tissue homogenate in saline (Potter-Elvehjem glass homogenizer).

Results

Clinical Findings

Each animal with a positive transplant died with clinical and morphologic evidence of leukemia. The first signs of a take were symmetrical abdominal enlargement or paralysis of the posterior extremities. Prior to death the animals showed weight loss and labored breathing. At the time they were killed, through 210 days post injection, about 5 percent of the animals that received injections of leukemic whole blood showed no gross or hematologic evidence of leukemia.

The average survival time of animals with positive transplants in 6 experiments was 42 days, with a range of 38 to 57 days. Of 83 rats injected and weaned, 79, or 95 percent, showed positive transplants.

Hematologic Findings

Animals were considered leukemic if peripheral-blood smears contained 1 percent or more myelocytes (*3*); white blood cell counts ranged from 6800 per mm.^{3} to 465,000 per mm.^{3} Differential white cell counts showed an increase in myelocytes and polymorphonuclear leukocytes, and the presence of normoblasts and degenerating cells during the last 10 days of life of the animal. Most rats from which serial white blood cell counts were obtained showed a progressive increase in white blood cells prior to death.

Gross Pathology

The tumor mass generally infiltrated the mesentery and involved the perirenal fat tissue. There was no apparent extension of the tumor from the perirenal fat into the kidney. The thymus and lungs were involved with metastatic tumor in that sequence. Metastatic lesions were also identified as poorly defined nodules in the liver, kidney (figs. 1 and 2), brain, and spleen in that order. The spleen on rare occasions revealed nodules that were identified by a green color. There did not seem to be a preferential tissue that encouraged growth of the tumor, but the neoplasm appeared more abundant and luxuriant in fat tissue. The metastatic lesions not readily visualized by the green color were easily recognized by a brilliant red color when illuminated (sensitized) with ultraviolet light. The fluorescent characteristics made it possible to identify metastasis that otherwise remained unrecognized by white light.

Microscopic Pathology

Supravital examination of tissue imprints revealed individual cells that did not appear to be a syncytium. The cells flowed freely in the serum suspension. The leukemic cells were pleomorphic, round in shape, and had a basophilic cytoplasm showing variations in density. There were fine and large mitochondria in the cytoplasm of most cells; a few contained refractile bodies and fine orange vacuoles. The nuclei were irregular in size and showed varying degrees of thickening of the nuclear membrane, with irregular nodularity. The nucleoplasm was basophilic and showed condensation of the nuclear chromatin. There was a large, centrally located nucleolus present, with some evidence of fine fibrillary extensions from the periphery of the nucleoli.

The sections stained with hematoxylin and eosin revealed the cells to be pleomorphic and showed frequent mitoses. The cytoplasm was acidophilic and somewhat granular. The nuclei were irregular in size and vesiculated, and nuclear condensations along with an irregular nucleolus were noted in most cells (fig. 3).

The chloroleukemic cells infiltrated along tissue planes and were appar-

eatly not limited by organ serosal surfaces. There was microscopic evidence of capsular invasion in such organs as spleen, kidney (fig. 2), and lymph nodes. The leukemic cells invaded the stroma in both skeletal and cardiac muscle. Microscopic examination of lymphoid structures revealed the presence of chloroleukemic cells, active phagocytes filled with red and white blood cells, cellular debris, nuclear debris, and altered blood pigment. These resembled the ovoid phagocytes reported by Shay *et al.* (*4*) and Wilens and Sproul (*5*).

The lymph nodes, spleen, and bone marrow showed varying degrees of replacement by chloroleukemic cells. In several animals, tissue sections of the brain showed a perivascular infiltration of chloroleukemic cells. The bone marrow showed complete replacement by leukemic cells in animals dying as a result of the leukemic process. Peroxidase stains with benzidine precipitations showed moderate reaction in normal bone-marrow preparations and a marked reaction in bone-marrow preparations from chloroleukemic rats and tumor imprints (fig. 4).

Transplantation Findings

Initially, successful transplants were made in suckling rats with 0.2 ml. of leukemic whole blood. Subsequently, successful transplants of the chloroleukemia could be obtained with 0.1 ml. or less of the inoculum. Results of the injection of dilutions of leukemic blood in normal plasma are shown in table 1. These data indicate that 0.0003 ml. of leukemic whole blood will produce 100 percent positive transplants. The reduction in the volume of injected leukemic whole blood increased the lifespan to 66 days. The investigation of this factor continues, and serial dilutions of cell numbers rather than volume are being done to determine the minimum number of cells necessary to transplant the chloroma. The effect of the dilution factor in the incidence of tumor occurrence and the relationship to survival time are being analyzed.

Table 2 shows that homogenates of tissue from leukemic rats produce successful transplants. However, in view of the dilution studies, which indicate that successful transplants can occur with minute amounts of blood, the successful transplants from tissue homogenates may be due to the small amount of blood in the tissues.

TABLE 1.—*Incidence of positive chloromyeloid leukemia transplants as a function of concentration of leukemic whole blood*

Amount of heparinized leukemic whole blood injected (*ml.*)	Number of injected rats	Number of rats developing chloroma	Age at death (*days post injection*)
0.2	17	16	45
0.1	40	38	44
0.03	4	4	57
0.01	12	12	56
0.003	14	13	60
0.001	18	18	61
0.0003	13	13	66

TABLE 2.—*Incidence of positive chloromyeloid leukemia transplants with tissue homogenates*

Material injected	Number of injected rats	Number of positive chloromyeloid leukemia transplants
Spleen homogenate	16	12
Chloroma homogenate	9	9
Kidney homogenate	6	5
Liver homogenate	5	5
Muscle homogenate	2	2

Results from an experiment comparing transplantability of intact cells and hemolyzed whole blood are given in table 3. It appears that whole blood or intact cells from centrifuged whole blood will produce a successful transfer of the tumor. Hemolyzed blood, its fractions, or leukemic plasma apparently will not result in a successful transplant. When leukemic whole blood was subjected to repeated freezing in an acetone dry-ice bath and thawing at room temperature (4 times), its leukemogenic activity was destroyed.

TABLE 3.—*Effect on transplantability of hemolyzed and intact leukemic blood*

Material injected (*ml.*)	Number of injected rats	Number of rats developing chloromyeloid leukemia
Intact whole blood, 0.1	6	5
Cells of spun whole blood, 0.1	8	6
Leukemic plasma, 0.1	6	0
Hemolyzed leukemic blood, 0.2	5	0
Ppt of spun hemolyzed blood, 0.1	6	0
Supernate-spun hemolyzed blood, 0.1	3	0

The effect of freezing and storing of leukemic whole blood at −20° C. for relatively long periods of time on transplantability is given in table 4. In view of the lability of transplantability by freezing and thawing, as shown in tables 3 and 4, experiments are under way to ascertain whether an intact cell is required for transplantation. To date a successful cell-free extract prepared according to the procedure of Gross (*6*) from leukemic

TABLE 4.—*Effect on transplantability of freezing whole blood*

Material injected (*ml.*)	Number of injected rats	Number of positive chloromyeloid leukemia transplants	Age at death (*days post injection*)
Fresh leukemic whole blood, 0.2	6	6	46. 4
Whole blood frozen 4 days, 0.2	4	0	
Whole blood frozen 20 days, 0.2	3	0	
Whole blood frozen 28 days, 0.2	2	0	

rats has not been obtained that will initiate the chloromyeloid leukemia in recipient suckling rats through 280 days post injection.

Table 5 indicates that the age of the rat at injection has an effect on acceptability of the rat to transplant. All rats 1 month old that received injections of 0.1 ml. of leukemic whole blood rejected the transplant. A smaller percentage of rats 2 weeks old rejected the transplant. One hundred percent of rats 1 week old or less accepted the transplant. Life-span of the animals increased with increasing age at injection.

TABLE 5.—*Effect of age of rats on susceptibility to transplantation*

Age of rats at injection	Number of injected rats	Number of positive chloromyeloid leukemia transplants	Age at death (*days post injection*)
1 month	23	0	
2 weeks	20	13	79
1 week	9	9	42
1 to 3 days	16	16	36

Discussion

There have been several reports of spontaneously occurring transferable leukemia in rats and mice as reviewed by Shay *et al.* (*7*). However, the transferable chloroleukemia in Wistar rats which had been induced by the gastric instillation of methylcholanthrene by Shay *et al.* (*3*, *7*) represents the first instance of a transferable chloroleukemia that has been successfully maintained through many subtransfers. The transplantable chloroleukemia in Sprague-Dawley rats reported herein, then, represents another system which has been maintained for many years.

Although these 2 chloroleukemias were induced by different types of carcinogens, their properties appear similar. The massive infiltration of tumor cells with green coloration, characteristic red fluorescence when examined under ultraviolet light, and high peroxidase content, is reminiscent of that reported by Shay *et al.* (*7*) and Schultz *et al.* (*8*). It has been shown that the chemically induced transplantable chloroleukemia in rats responds to triethylene thiophosphoramide (*4*). Similar studies are under way in our laboratory to assay the chemotherapeutic response in what may be considered a radiation-induced transplantable chloroleukemia. Investigations are being expanded to define more clearly the similarities or dissimilarities if they exist. For, if these 2 chloroleukemias are identical, then one can assume that the response produced is independent of the nature of the etiologic factor in inducing leukemia in the rat. Similarly, any differences in the biologic properties manifested by the radiation-induced chloroleukemia may be indicative of a unique response of tissue to actinium irradiation.

Initially, transplants were made with 0.1 to 0.2 ml. of leukemic whole blood; however, as little as 0.0003 ml. of leukemic whole blood produced positive transplants. More recently, it was found that successful transfers

resulted from intraperitoneal injections of 1×10^3 white blood cells. The differential count on this sample was 9 percent myelocytes, 9 percent stabs, 40 percent polymorphonuclear leukocytes, and 42 percent lymphocytes. There was no attempt to differentiate or determine the number of viable cells transferred. The injection of reduced numbers of cells increased longevity from 42 days when 6×10^6 cells were injected to 74 days when 1×10^3 cells were injected. When between 5×10^4 and 1×10^3 cells were injected, the percentage of positive transplants fell from above 90 percent to about 40 percent. Further studies are being made in an attempt to quantitate the viable myelocytic count with the percentage of positive transplants and the onset of the chloroleukemia and date of death.

Strikingly different results were obtained when 2 samples of leukemic whole blood were injected, containing approximately equal numbers of white cells but differing number of myelocytes. The sample with the greater myelocyte count produced an earlier onset of chloroleukemia and death. Shay *et al.* (7) noted this apparent dependence of transplantability of blood upon myelocyte count in their experiences with a transplantable rat chloroma.

Tissue homogenates of various organs and tissues from chloroleukemic rats, when injected into host suckling rats, produced chloroleukemia as did whole blood. The relatively small number of cells necessary to produce a positive transplant suggested that the residual blood in the tissue homogenates was responsible for the development of chloroleukemia. These studies offer assurance that selected tissues have no enhancing or inhibiting effect on the transferability of chloroleukemia.

Experiments with freezing and thawing and storage of leukemic whole blood, as well as our inability to produce positive transplants with cell-free filtrates, cause us to conclude at this time that intact cells are a prime requisite for transferability.

Harris *et al.* (9) have shown with their rat chloroleukemia that successful transfer by cell inoculation is due to colonization of donor cells in the recipient animal and is not induced by leukemogenic cell particles or filterable agents. We interpret our experiences as being in consonance with this concept, which implies that the host serves only as a milieu for colonization.

Comparison of the transplantable chloroleukemia made in 1958 with that made in 1954 showed the morphologic characteristics, when examined histologically by supravital stains and phase microscopy, to be identical (figs. 3 and 5). The transplantability under standard conditions of injection (0.2 ml. of whole blood into young rats) has remained the same; the average date of death and percentage of positive transplants have neither increased nor decreased.

To our knowledge, the spontaneous occurrence of chloroleukemia in Sprague-Dawley rats has not been reported. The occurrence of chloroleukemia in 2 rats following injection of actinium-227 (AEM) implies a causal relationship between actinium as an internal emitter and chloro-

leukemia. It is of interest to point out that in our experiences with many thousands of rats injected with polonium-210, a pure α-emitter, with exposures up to 720 days, no myelogenous leukemia developed. However, polonium-210 was found after injection in relatively high concentration in the soft tissues, whereas actinium-227 (AEM) has a relatively high concentration in bone with a specific affinity for the endosteum and periosteum.

It is likely that this predilection for endosteum and periosteum by actinium, with its associated α-, β-, and γ-emitting radiation spectrum, is the causative factor for leukemogenesis.

The potential usefulness of this chloroleukemia, assumed to have been incited by actinium-227 (AEM), can only be developed by further exploration of the characteristics of the immature granulocytes. There is a possibility that this chloroleukemia, which appears morphologically similar to that described by Shay *et al.* (*3*) and induced by methylcholanthrene, may have a different metabolic pattern and react biologically to similar test systems in a different manner. The application of this system to various test procedures may elucidate a reaction comparable to that in man.

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PLATES

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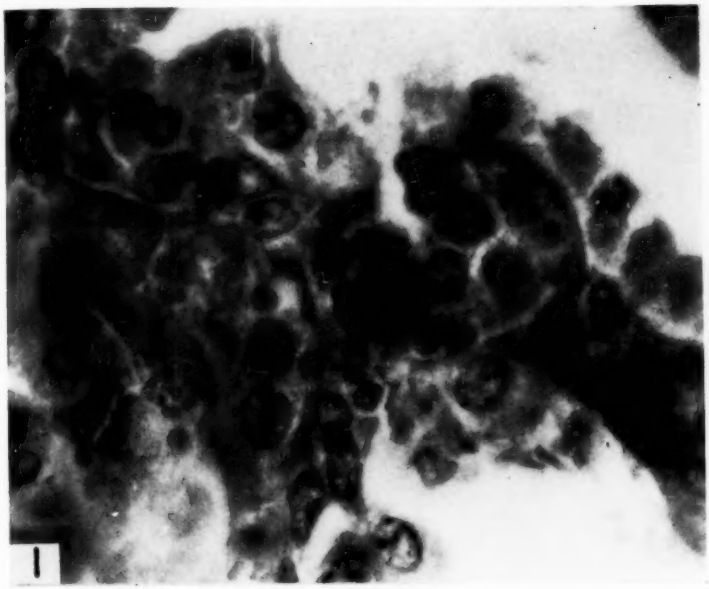
PLATE 68

FIGURE 1.—Chloroleukemia in rat kidney. *Note* infiltration of chloroma into the parenchyma of kidney about the collecting tubule. *Note* mitosis. × 1,500

FIGURE 2.—Chloroleukemia in rat kidney capsule. *Note* infiltration of kidney with invasion of the capsule. × 1,500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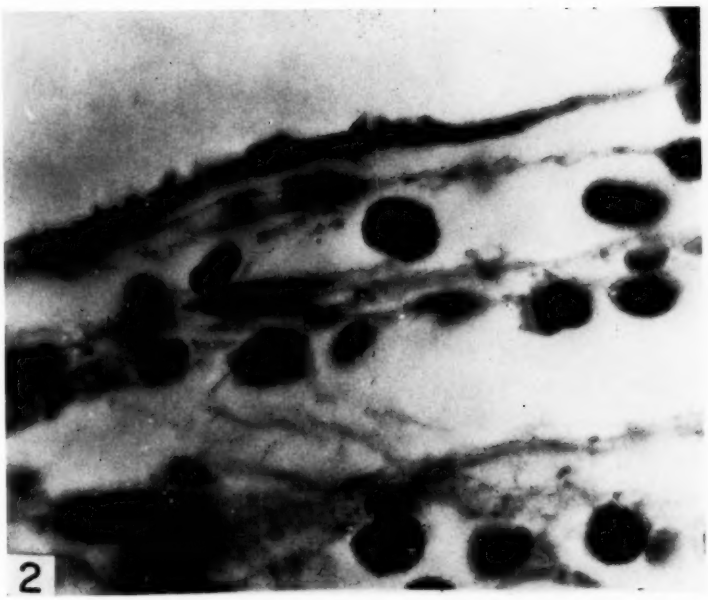
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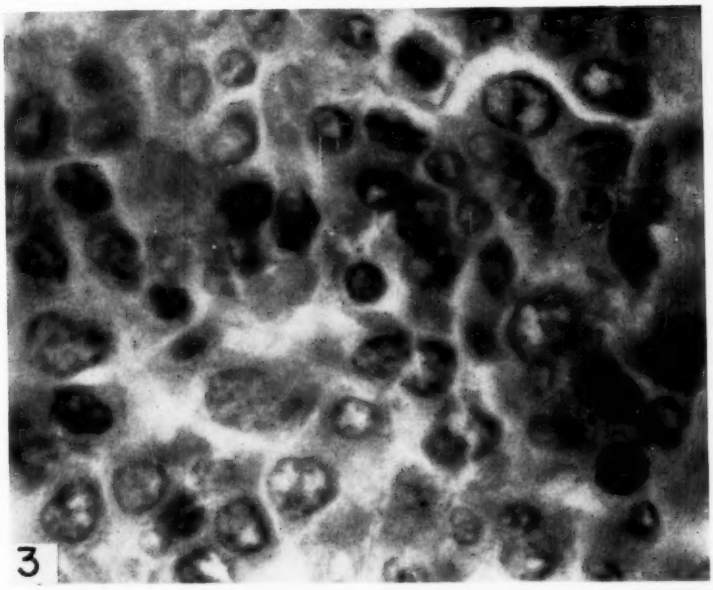
PLATE 69

FIGURE 3.—Section of lymph node from chloroleukemic rat taken in 1954. *Note* confluent infiltration and mitosis. × 1,500

FIGURE 4.—Tumor imprints from chloroleukemic rat showing well-marked benzidine precipitation. × 1,500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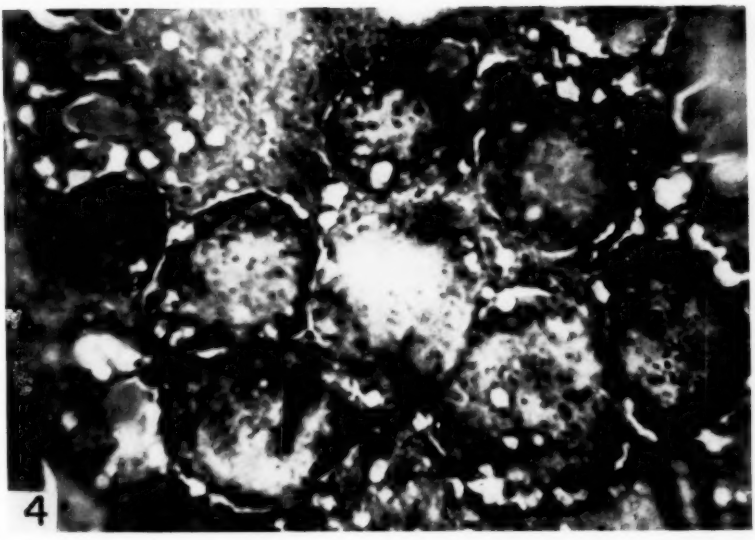
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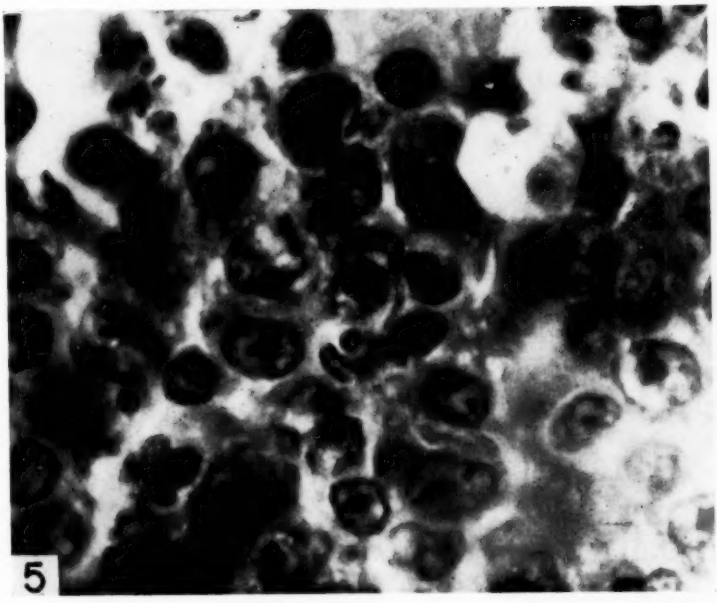
PLATE 70

FIGURE 5.—Section of lymph node from chloroleukemic rat taken in 1958. *Note* the identical histologic and morphologic characteristics when compared with figure 3. × 1,500.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Stimulation of Appetite and Growth in the Tumor-Bearing Rat by a Variety of Tissues[1]

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Summary

Sprague-Dawley rats bearing the Walker carcinosarcoma 256 subcutaneously and maintained on a 25 percent casein diet (C-25) were transferred, after weight loss, to a variety of diets in which lyophilized tumor tissue (rat, mouse, or human) or normal rat tissue (liver or muscle) replaced casein. Under these conditions, animals ingesting the tumor diets showed variable weight gains. A diet (R_{NH}) containing tissue of the Novikoff rat hepatoma was similar to diet T-24 in producing large weight gains and increases in food intake. Diets containing defatted human tumor and defatted Walker 256 tumor gave equal growth response. Animals transferred to normal muscle or liver diets showed average increases of 2 to 3 gm. per day, considerably less than animals transferred to T-24, which averaged 11 gm. per day. Tumor-bearing rats on C-25 similarly transferred, after weight loss, to a 20 percent casein diet (C-20), or allowed to remain on C-25, showed a smaller positive response than rats transferred to T-24. Rats bearing the Novikoff hepatoma subcutaneously were transferred to T-24 after weight loss on C-25, and weight-loss reversal was observed whenever the diet was acceptable. R_{NH}, T-24, and C-20 were compared for their effect on body-weight change and tumor size in Novikoff hepatoma-bearing rats fed these diets during the period of tumor growth. Both tumor diets produced far greater increase in total body weight and in carcass-weight change than C-20 did. Tumor weight was greater for rats on the T-24 diet than for those on C-20, but was intermediate for rats on R_{NH}, showing greater variability.—**J. Nat. Cancer Inst. 22: 685–696, 1959.**

Rats bearing the transplanted Walker carcinosarcoma 256 and maintained on a 25 percent casein diet showed an abrupt decrease in both weight and food intake. Such animals transferred to a diet containing lyophilized Walker 256 tumor in place of the casein responded with an immediate and a large increase in food intake and body weight (*1*). Furthermore, the appearance of larger tumors and of smaller carcass-weight loss in rats maintained on the tumor diet suggested that the tumor

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

tissue in the diet may have altered the tumor-host relationship in that depletion of carcass tissue, ordinarily a concomitant feature of tumor growth, had been reduced.

To determine whether the effect on body weight and food intake was characteristic of other tumor tissues and thus of cancer, in general, experiments are reported here in which 1 rat, 3 mouse, and 3 human tumors were similarly studied.

Materials and Methods

Preparation of diets.—The composition of the 2 casein control diets, C-25 and C-20 (20 percent casein), has been given previously (*1*). These 2 diets contained protein, fat, carbohydrate, vitamin supplements, and salts adequate for the maintenance and growth of normal rats. The C-20 diet contained an additional 0.5 percent methionine which was not in C-25. All the experimental diets were similar to C-20 but contained lyophilized normal or tumor tissue as a source of nitrogen in place of casein. The percentage of tissue used yielded a diet with a nitrogen content approximately equal to that of C-20 (about 30 mg. N/gm. diet). The starch content of each diet was altered to compensate for any such adjustment in tumor-tissue content. All diets were essentially isocaloric.

Defatted (lipide-free) tumor material was used in 2 diets. Fat-free Walker tumor was prepared by treating lyophilized tumor tissue with 2:1 alcohol-ether followed by 1:1 chloroform-methanol and then by additional alcohol-ether to remove the residual chloroform. This fat-free material has been found to be equivalent to whole dry Walker 256 tumor tissue in its effect on weight gain in tumor-bearing rats (*2*, *3*). In the present instance, the fat-free material was used as a control for one human tumor which was also defatted.

A summary description of the diets is given in table 1. Two simple rules were used in naming all diets other than those composed of casein or the Walker carcinosarcoma 256: 1) The main letter of each diet reference term designated the specific source of the tissue: R = rat, M = mouse, H = human. 2) The subscript letter referred to the kind of tissue or tumor: L = liver, M = muscle, NH = Novikoff hepatoma, S = sarcoma, A = adenocarcinoma, F = fibrosarcoma, C = carcinoma, R = rhabdomyosarcoma. The designations of the 2 casein diets, C-20 and C-25, and the whole Walker tumor diet, T-24, were those used previously (*1*). The other Walker tumor diet, containing defatted tumor tissue, was called T-22.

Feeding experiments.—The methods for testing the casein control diets, the normal tissue diets, and the tumor-tissue diets were the same as those described previously (*1*). The test animals were male Sprague-Dawley rats which, at 150 to 200 gm., were inoculated subcutaneously with the Walker carcinosarcoma 256, except for 2 groups which received the Novikoff hepatoma. These rats were maintained on a Purina chow diet before the experiments began. They were housed individually in suspended wire cages, and given food and water *ad libitum* when placed on

the experimental diet. Food consumption and weight change were recorded daily.

Experiments were carried out on Walker tumor-bearing rats placed on C-25, 3 to 11 days before tumor inoculation, and thereafter individually observed for weight loss and decreased food intake. When these changes occurred, the rats were transferred to any one of the experimental regimens, remaining on the particular diet until they either died or were killed because their tumors had ulcerated. One group of rats was allowed to continue on the C-25 diet until the animals either died or had to be killed.

In regard to the 2 defatted tumor diets, H_{C_2} and T-22, tumor-bearing rats were first maintained on the C-20 diet rather than on C-25. In our experience, the behavior of tumor-bearing rats fed C-20 has been more

TABLE 1.—*Description of diets*

Material incorporated into diet	Diet reference term	Material incorporated (*percent*)
Casein	C-20	20
Casein	C-25	25
Normal rat liver	R_L	28
Normal rat hind-leg muscle	R_M	25
Walker rat carcinosarcoma 256	T-24	24
Walker rat carcinosarcoma 256 (defatted)	T-22	22
Novikoff rat hepatoma	R_{NH}	24
Mouse sarcoma 37*	M_S	32
Mouse adenocarcinoma C3HBA†	M_A	24
Mouse fibrosarcoma H-38290†	M_F	24
Human carcinoma #1‡	H_{C_1}	28
Human carcinoma #2 (defatted)‡	H_{C_2}	22
Human rhabdomyosarcoma‡	H_R	24

*Supplied by Mr. Adrian Perrault, Laboratory of Chemical Pharmacology, National Cancer Institute.

†Transplantable tissue of the adenocarcinoma and fibrosarcoma were provided, respectively, by Dr. Morris K. Barrett, Laboratory of Biology, and Dr. Richard L. Swarm, Laboratory of Pathology, National Cancer Institute.

‡Supplied by Dr. Louis B. Thomas, Pathologic Anatomy Branch, National Cancer Institute.

predictable and uniform than those fed C-25. The animals on the C-20 diet consistently tended to reach a weight plateau 20 to 25 days after transplant, while animals fed C-25 might show large weight losses, but occasionally only small losses. A number of animals, which had reached their weight plateau on the C-20 diet and remained at that level, were transferred to H_{C_2} or T-22.

Ulceration of the transplanted tumors appeared in many animals, ranging from 20 to 60 percent in any given group. Such animals might show loss of weight and lowered food intake regardless of tumor size. Thus all animals showing any signs of ulceration during the early stages of these experiments were discarded and are not included in the data. The method

of assay used was not too desirable because of the consistent appearance of ulcerated tumors.

All the feeding experiments described so far dealt with rats bearing the Walker carcinosarcoma 256. Since it was important to determine whether other transplanted tumors responded in a manner analogous to the Walker tumor, 2 additional studies were made with rats bearing the subcutaneously transplanted Novikoff hepatoma. In one experiment, rats bearing this tumor were maintained on the C-25 diet, observed for weight loss and decreased food intake, and then transferred to the T-24 diet.

Another experiment was carried out on the response of the Novikoff hepatoma to tumor diet fed continuously during the period of tumor growth. Three groups of animals bearing this tumor subcutaneously were fed C-20, T-24, or R_{NH}, respectively. This regimen was begun on the 7th day following transplantation and was continued for 22 days.

Results and Discussion

Table 2 illustrates some of the results obtained with rats bearing the Walker tumor. The total period over which each rat was studied has been divided into 3 separate periods whose lengths were determined by the behavior of each rat. Period A covers the time from tumor transplantation until the date when maximum weight was reached by the rats on the C-25 diet. Period B is a continuation of period A with the rats on C-25 but covers the time when the animals were losing weight. Period B extends to the time when the rats were removed from the C-25 diet and transferred to one of the diets containing tumor or normal tissue or to C-20. Period C covers the remaining time until death of the animals. When the supply of diet was limited, period C was also limited. On the other hand, when tumor tissue was plentiful, period C could be comparably long. Since, in our experience, response to T-24 has been immediate, there were times when period C was arbitrarily cut short after a week. It should be emphasized that the division between periods A and B serves mainly to show the change in the growth pattern of the animal.

In a comparison of the behavior of animals on these different diets, it is first important to point out that the groups of rats indicated in the table were chosen for these tests from stock animals at various times over a period of 15 months as the experimental materials became available. During this time there was considerable variability in the behavior of animals on C-25. As their tumors became large, some animals showed large weight losses, whereas others showed a tendency to reach a maximum weight and remain at or somewhat below that level.

Most of the animals placed on any of the control diets (C-20, R_L, or R_M) showed a positive response in period C as compared with period B (table 2). However, the average weight change was 0.8, 2.0, and 3.5 gm. per day, respectively, compared to T-24 which gave a response of 11 to 12 gm. per day [table 2 and (*1*)]. Daily weight change for these 3 control groups was on the average slightly less when calculated for the first 5 days only of period C (table 2). Individual results have not been given for

the one control group allowed to remain on C-25 continuously. Of a total of 13 rats in the group, 9 lost weight for 3 to 9 days and then slowly regained weight at an average rate of 3.1 gm. per day. The remaining 4 rats continued to lose weight until death.

Animals which ingested the tumor diets showed variable positive responses. (Failure to eat a diet resulted in continued weight loss.) Rats on the R_{NH} diet showed the best response, comparable to that obtained with T-24. It is apparent that food intake alone cannot account for the very large weight gains by the T-24 and R_{NH} groups. Accompanying these increases in food intake were increases in water consumption. Animals ingesting the mouse tumors showed a response equal in most cases to about half that observed with T-24. The human tumors reported in this table showed variable results. All rats ingesting H_R showed weight gains, varying from 30 to 90 percent of the response on T-24. Of 7 animals on H_{C_1}, 3 showed excellent response and 4 failed to respond at all.

As mentioned earlier, the method of biological assay in these experiments was far from ideal. Variability in the condition of the animals on C-25 prior to transfer to one of the other diets was unavoidable, for each animal responded in an individual manner to the impact of the tumor. The decision to transfer an animal from C-25 to one of the other diets was based on day-to-day observation of weight change, food intake, and appearance of the tumor. For example, an animal might show a large weight change (20–30 gm.) in a few days, which indicated the transfer was advisable at this particular time. The sudden cessation of food intake for a few days would also suggest transfer. On occasion, when bareness of the tumor accompanied either weight loss or decreased food intake or both, a rat was transferred to period C sooner than animals which did not show this change in appearance of the tumor.

In table 3, the effect of the 2 defatted tumor diets, H_{C_2} and T-22, is directly compared. Previous experience indicated that T-22 was equivalent to T-24 in stimulating growth and appetite (*3*). H_{C_2} was similarly equal to T-22. It should be noted that the weight loss of these animals during period B was small (table 3), which is a feature uniformly observed in animals on a 20 percent casein diet.

Weight change and food intake of rats bearing the Novikoff hepatoma and maintained at first on C-25 are presented in table 4.

The response of animals to the presence of the hepatoma during the period the rats were on C-25 differed markedly from that of animals bearing the Walker tumor. Of 38 animals originally designated for the experiment, 16 produced tumors which became bare or ulcerated before the rats were ready to be transferred to the T-24 diet. An additional 6 rats died suddenly without loss of weight before they could be transferred, and 3 rats were found dead the morning after they were changed to the tumor diet. This sudden death was observed quite frequently in our stock of hepatoma animals but was not so apparent among the Walker 256 tumor-bearing rats.

The response of the remaining 13 rats which ultimately were transferred

TABLE 2.—*Effect of normal tissue and tumor diets on weight change and food intake of rats bearing the Walker carcinosarcoma 256*

Rat No.	Period A			Period B			Diet fed during period C	Period C			Period C (first 5 days)	
	Days on C-25*	Average weight change (*gm./day*)	Average food intake (*gm./day*)	Days on C-25	Average weight change (*gm./day*)	Average food intake (*gm./day*)		Days on diet	Average weight change (*gm./day*)	Average food intake (*gm./day*)	Average weight change (*gm./day*)	Average food intake (*gm./day*)
337	22	5.2	10.1	4	−10.0	1.0	C-20	27	2.3	7.0	6.2	7.6
340	24	5.7	10.9	5	−5.6	2.6		9	1.7	7.2	0.6	6.4
328	23	5.1	9.9	5	−6.2	2.4		13	1.6	6.5	3.4	7.4
334	23	6.2	12.5	5	−8.4	1.6		27	1.0	6.6	−3.8	1.8
336	17	5.4	10.9	5	−4.8	2.8		20	0.7	5.5	−3.6	2.0
354	17	5.3	9.8	5	−5.2	1.8		23	0.6	5.2	−3.2	2.8
342	17	5.6	11.1	6	−5.7	2.5		13	0.5	5.9	3.4	6.4
326	20	6.0	11.8	4	−7.0	1.3		26	0.3	5.2	−9.0	0.6
346	21	6.2	12.1	9	−5.1	3.2		11	0.1	7.1	3.0	8.0
348	17	5.7	11.4	6	−4.5	2.7		12	0	5.3	−2.6	4.0
327	30	5.2	10.5	5	−6.6	1.8		13	−0.5	5.2	−3.0	3.8
3118	15	5.5	10.9	11	−1.5	4.2	R_L	7	5.4	8.3	5.6	8.8
2016	20	1.5	7.3	9	−5.7	1.1		10	4.7	6.7	5.4	6.4
2020	22	5.6	9.9	6	−7.2	2.0		9	3.2	6.8	4.8	5.8
3119	26	5.6	11.0	6	−3.3	3.8		7	2.6	8.7	3.0	8.3
2015	20	4.5	10.0	5	−8.6	0.2		10	2.3	5.1	0.8	4.4
3135	20	4.6	9.1	6	−2.0	3.8		7	2.1	5.9	1.2	5.4
3139	15	4.9	10.3	11	+0.5	5.0		7	1.1	5.0	0.4	4.6
3121	26	5.1	9.6	6	−0.2	6.3		7	−1.3	4.4	−2.6	3.6
3147	21	5.6	10.3	5	−1.6	4.4		7	−1.9	3.0	−4.2	2.0
3105	20	3.8	8.2	6	+0.3	5.2	R_M	7	4.9	7.9	3.6	7.8
3110	25	3.1	7.8	7	+0.9	6.6		7	4.6	8.4	4.2	8.3
3113	11	5.5	10.9	15	−1.7	4.0		7	4.3	7.3	4.6	7.8
3143	25	5.9	11.8	4	−6.0	2.5		10	4.1	7.0	2.8	6.0
2043	18	3.0	9.2	7	−3.9	2.3		10	4.0	6.6	3.8	6.2
3145	18	5.5	9.9	8	−1.8	4.0		7	3.7	6.9	3.0	6.8
2012	20	4.7	9.7	7	−4.7	2.1		12	2.9	6.0	2.8	6.0
3127	18	5.3	9.2	8	−0.6	4.8		7	2.3	6.3	2.2	6.0
3132	25	5.8	10.2	7	−3.0	3.6		7	0.3	5.6	−1.1	5.0
3141	15	5.0	11.3	11	−2.0	3.5	T-24	7	12.0	8.4		
3124	18	2.7	8.6	8	−1.4	4.1		7	11.6	7.7		
3103	18	4.9	9.7	8	−0.9	3.5		7	9.9	7.7		
40	25	5.7	12.8	5	−6.0	1.8	R_{NH}	4	28.8	17.8		
38	30	6.2	11.9	3	−10.7	0.3		3	24.3	14.3		
	33	26	5.2	11.2	6	−4.3	4.3		5	23.0	13.6		
								6	16.8	11.8		

38	30	6.2	11.9	3	−10.7	0.3	H_{NR}	3	28.8	17.8		
								3	24.3	14.3		
33	26	5.2	11.2	6	−4.3	4.3		5	23.0	13.6		
30	26	5.6	12.1	3	−9.7	1.3		6	16.8	11.8		
41	29	5.2	11.3	7	−3.9	3.4		9	16.0	10.4		
73	20	4.5	11.2	5	−7.2	1.6		4	14.8	12.2		
34	26	6.2	12.7	6	−6.2	4.2		10	14.3	9.2		
32	28	6.5	11.9	3	−7.7	3.0		11	13.7	9.7		
36	26	5.0	11.7	5	−5.8	2.2		6	13.2	9.2		
62	17	5.0	10.6	6	−6.3	1.7		15	12.2	10.3		
72	17	5.2	11.1	6	−4.2	2.7		15	7.6	7.7		
74	17	4.6	10.2	6	−3.8	3.2		8	7.4	8.8		
71	17	5.5	11.6	5	−5.2	3.6		15	7.0	9.7		
70	13	5.0	11.3	7	−5.7	2.0		18	6.1	9.7		
35	22	4.6	9.0	13	−2.4	3.8		3	−4.7	2.7		
39	25	5.0	10.9	4	−10.8	1.8		2	−12.5	0		
331	14	4.9	10.2	7	−3.4	2.6	M_S	3	8.7	7.3		
333	16	5.4	11.6	5	−6.0	0.8		3	8.0	7.3		
345	11	5.1	10.6	10	−5.6	1.8		3	6.0	5.0		
330	14	5.6	11.1	7	−4.4	3.0		2	−5.0	0		
343	16	4.4	9.8	5	−9.0	1.0		2	−6.0	0		
3501	26	4.5	9.8	3	−1.3	5.0	M_A	6	8.7	7.2		
3435	25	4.1	9.1	2	−1.0	4.5		6	7.5	7.3		
3441	26	4.8	10.1	5	−0.2	5.6		7	5.6	6.3		
3406	24	3.9	9.9	3	−0.7	5.3		9	5.3	7.1		
3442	23	5.3	10.4	2	−3.0	4.5		7	1.0	3.7		
3405	20	4.1	9.2	5	−0.4	5.5	M_F	7	7.7	8.0		
3408	27	4.8	9.0	5	−0.2	6.2		7	7.3	8.4		
3500	20	5.4	10.4	5	−4.4	2.6		11	6.6	7.0		
3493	26	5.2	10.8	3	−3.7	4.0		4	5.5	7.2		
3411	23	5.1	9.8	4	−1.0	5.0		9	3.6	5.2		
430	18	6.3	12.4	11	−6.7	1.6	H_{C_1}	3	16.0	9.0		
415	24	5.7	13.0	5	−10.2	4.6		3	10.7	5.7		
421	24	5.2	10.7	4	−7.0	1.7		8	6.8	6.2		
423	17	4.8	10.6	4	−7.0	0.2		2	−5.5	1.5		
424	22	6.6	12.8	6	−9.3	1.1		1	−8.0	0		
422	15	7.0	13.3	6	−10.1	0.7		2	−8.5	0		
416	21	6.4	12.0	7	−10.3	0.8		1	−14.0	0		
3410	23	5.4	10.0	4	−2.8	4.2	H_R	9	11.1	8.0		
3495	18	3.3	8.3	7	−2.0	4.0		10	5.4	6.0		
3418	15	3.1	9.6	6	−5.2	1.5		8	4.6	6.0		
3503	27	4.3	8.8	2	−2.0	4.0		7	3.6	5.4		

*From time of tumor transplantation.

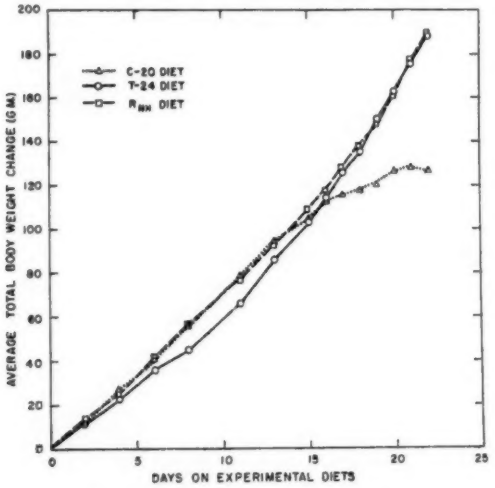
TABLE 3.—*Effect of 2 defatted tumor diets on weight change and food intake of rats bearing the Walker carcinosarcoma 256*

Rat No.	Period A			Period B			Tumor diet fed during period C	Period C		
	Days on C-20	Average weight change (*gm./day*)	Average food intake (*gm./day*)	Days on C-20	Average weight change (*gm./day*)	Average food intake (*gm./day*)		Days on diet	Average weight change (*gm./day*)	Average food intake (*gm./day*)
1649	14	3.5	8.1	9	−0.8	4.6	HC_2	5	7.2	7.8
1635	14	5.6	10.5	9	−4.2	3.1		5	7.0	8.8
1598	14	4.4	8.1	7	−1.4	5.0		7	6.3	7.5
1650	19	5.3	10.6	5	−2.0	5.4		8	5.4	8.1
1648	10	3.4	7.9	13	−0.8	4.0		5	5.2	7.0
1600	17	4.9	8.9	4	+0.2	6.0		3	−4.7	2.0
1646	10	4.7	10.1	13	−1.4	4.9	T-22	5	9.6	8.6
1641	19	4.1	9.4	5	−2.6	3.8		8	7.2	8.0
1601	7	5.0	8.3	14	−0.1	5.2		7	5.3	6.9
1633	14	6.3	11.6	9	−2.9	4.0		4	4.8	7.5
1634	19	5.6	11.9	4	−3.0	4.5		4	2.0	5.8

TABLE 4.—*Effect of T-24 on weight change and food intake of rats bearing the Novikoff hepatoma*

Rat No.	Period A			Period B			Period C		
	Days on C-25	Average weight change (*gm./day*)	Average food intake (*gm./day*)	Days on C-25	Average weight change (*gm./day*)	Average food intake (*gm./day*)	Days on T-24	Average weight change (*gm./day*)	Average food intake (*gm./day*)
278	25	5.5	10.3	2	−3.5	4.5	2	26.5	18.0
282	28	5.8	10.7	2	−5.5	2.5	1	21.0	15.0
293	26	6.8	12.3	1	−20.0	0	3	19.0	12.0
281	23	5.5	10.4	4	−1.0	5.5	3	16.3	10.7
267	30	5.8	11.4	3	−3.3	5.0	1	15.0	15.0
298	33	5.1	11.5	1	−11.0	1.0	1	13.0	16.0
276	21	6.6	12.5	6	−2.7	5.5	7	8.7	9.7
286	25	6.2	10.5	2	−4.0	6.0	4	2.8	5.0
271	22	6.8	11.5	5	−3.8	4.8	2	2.5	10.0
280	28	6.4	11.9	3	−1.3	6.3	1	−5.0	7.0
277	21	6.3	10.6	6	−6.3	4.7	1	−7.0	0
294	21	5.6	11.0	2	−8.5	2.0	3	−9.3	0.7
291	21	8.0	14.0	2	−13.0	0	3	−11.0	0.7

to T-24 varied considerably (table 4). It will be noted that the length of period C for these rats was shorter than that for the rats of the groups in table 2. All the hepatoma-bearing rats showing positive response to T-24 (table 4) had to be killed because of tumor ulceration, and thus some could only be on the T-24 diet for 1 day. The remaining animals ate little or no tumor diet, continued to lose weight, and died in 3 days or less after being transferred from C-25 to T-24. The high percentage of deaths is not surprising in view of the fact that the hepatoma-bearing rats on many occasions died even without loss of appetite. Four of the 7 nonresponding rats showed paralysis of the hind quarters a few days before death. Apparently the Novikoff hepatoma is capable of producing such a sudden lethal effect in the host that positive response by the animal to the tumor diet cannot always be demonstrated. Where positive responses did occur



TEXT-FIGURE 1.—Total body-weight change of Novikoff hepatoma-bearing rats ingesting either the C-20, the T-24, or the R_{NH} diet.

(table 4), the increases were often comparable to those observed in rats bearing the Walker tumor and transferred from C-25 to R_{NH} (table 2).

The results of feeding C-20, T-24, or R_{NH} to hepatoma-bearing rats during the final 22 days of tumor growth are shown in text-figure 1 and table 5. Weight gain by rats on either of the 2 tumor diets was strikingly greater for the final 6 or 7 days before death than the weight gain by animals on the casein diet (text-fig. 1). It must be noted, however, that food intake of the latter animals during the last week was somewhat lower than that of rats on either of the tumor diets. This point is demonstrated in table 5 where mean weight change and food consumption (both calculated as gm./day) are given for the first 16 days, on the diets, and for the last 6 days. Also shown are the mean initial weights and the mean over-all weight changes for each of the 3 groups for the 22-day period.

TABLE 5.—*Food consumption and weight changes (tumor and carcass) of Novikoff hepatoma-bearing rats ingesting either the C-20, the T-24, or the R_{NH} diet (mean ± standard error)*

Diet	Number of rats	Initial body weight (*gm.*)	0 to 16 days		17 to 22 days		0 to 22 days		
			Total body-weight change (*gm./day*)	Food intake (*gm./day*)	Total body-weight change (*gm./day*)	Food intake (*gm./day*)	Total body-weight change (*gm.*)	Tumor weight (*gm.*)	Carcass-weight change (*gm.*)
C-20	8	204±6	7.1±0.4	13.0±0.4	2.2±1.0	8.8±0.9	126±10	101±5	25±8
T-24	8	205±5	7.2±0.4	11.6±0.3	12.4±0.8	11.2±0.6	189±7	131±7	58±10
R_{NH}	10	204±3	7.4±0.4	12.9±0.4	11.8±0.6	11.1±0.3	188±8	119±15	69±10

Carcass-weight change has been calculated as total body-weight change less tumor weight.

During the first 16 days, growth by all 3 groups was essentially the same despite a somewhat diminished food intake by animals on T-24. In contrast, during the last 6 days of the experiment, weight change by rats on either tumor diet, T-24 or R_{NH}, was some 5 to 6 times greater than weight change on the casein diet. As for the weight changes over the entire 22-day period, the total body-weight change was the same for rats on either of the 2 tumor diets and about 50 percent greater than that of the casein controls.

The variation in size of the tumors as a result of the R_{NH} diet was large—the standard error of the mean being about twice that observed for tumors growing in rats receiving the other 2 diets. One rat from the R_{NH} group had an unusually small tumor which continually lagged behind the others during the period of growth. This single weight lowered the mean tumor weight of the group and increased the standard error. If this animal is excluded from the group, the mean total body-weight change is virtually the same as before, *i.e.*, 191 $\pm$ 8 gm. However, the mean tumor weight and carcass-weight change become 127 $\pm$ 11 gm. and 64 $\pm$ 10 gm., respectively. These figures are similar to the values obtained on the T-24 diet (table 5).

The rats in this experiment were maintained only 22 days on the experimental diets before being killed, to avoid loss of too many animals by ulceration and death. All but one rat showed positive carcass-weight changes at the end of the experimental period, but carcass-weight changes were larger on either tumor diet than on C-20. In the earlier studies with the Walker carcinosarcoma 256 (*1*) tumor-bearing rats were fed the T-24 or C-20 diet for 27 days. In that case, carcass-weight changes were nearly all negative, though again the tumor diet produced the more favorable carcass change. The fact that positive carcass-weight changes were observed with the Novikoff hepatoma, and negative changes with the Walker tumor, could be due to *a*) the difference in length of time of the experiment, *b*) a difference in the nature of the 2 tumors, or *c*) a combination of both factors. One can conclude from the present experiment, however, that the response of the Novikoff hepatoma and its host to either of the 2 tumor diets is qualitatively similar to that observed earlier with the Walker 256 tumor.

Fractionation studies on tumor tissue are now in progress to determine the component(s) responsible for the results reported here.

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Comparative Fluorescein Labeling and Cytotoxicity Studies With Human Cell Strains HeLa, Raos, and 407-Liver and With Fresh Surgical Specimens of Cervical Carcinoma, Osteogenic Sarcoma, and Normal Adult Liver[1]

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Summary

Antiserums prepared against a human melanoma and against normal human thyroid gave the same staining and cytotoxic reactions as antiserums to two permanent human cell lines, HeLa and 407-liver. The antibody, which was developed in rabbits, reacted primarily with connective tissues in noncultured surgical specimens. Antimelanoma serum showed a staining reaction with both HeLa and Raos cultures and with the tumor cells of cervical carcinoma and osteogenic-sarcoma tissues obtained from specimens for biopsy. This indicated that similar antigens are present in the tissue-culture cells as in the cells of the same type of tumor from which they were derived. Hepatic cells did not stain with the antiserum, although good staining could be demonstrated in cultures of 407-liver strain. This suggested that the liver strain may have originated from connective tissue rather than from embryonic parenchyma in the liver.—**J. Nat. Cancer Inst. 22: 697–705, 1959.**

Human cells grown in tissue culture show extensive immunological cross reactions so that it has not been possible to distinguish one cell strain from another by immunological methods. Mountain (*1*), Miller and Hsu (*2*), and Goldstein (*3*) have shown that cytotoxic antiserums which were prepared by inoculating rabbits with human cell strains were not specific but cross-reacted and were cytotoxic for other human cells tested in cultures.

Recently, Hiramoto, Goldstein, and Pressman (*4*) reported that antiserums prepared against HeLa and a strain of cells derived from human fetal-liver tissue reacted with supporting stromal elements of human thyroid, liver, kidney, and adrenals, as indicated by fluorescein-labeled antibody staining. The data showed that the tissue culture of human cells contained antigenic substances in common with the stromal elements of these tissues.

In the present study, experiments were performed to determine whether antibodies formed against homogenates of biopsy specimens of

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normal and malignant tissues were cytotoxic for human cells growing in tissue culture, and whether these antibodies, when labeled with fluorescein according to Coons' technique (*5*), would stain human cells, in culture and in biopsy specimens, like those from which the tissue-culture cells may have originated.

Two antiserums developed in rabbits were studied: one against normal human thyroid and the other against a human melanoma. These serums were chosen because they strongly stained human connective tissue with fluorescein-labeled antiserums as labeled antiserums did, which were prepared against HeLa and 407-liver and were described in a previous report (*4*).

The cultured cells were of two morphologically distinct, permanent strains of malignant origin, HeLa and Raos, and of a strain of epithelial cells (similar in morphology to HeLa) established from the liver of a fetus. The fluorescent staining of these cell strains was compared with that used for surgical specimens of cervical carcinoma, osteogenic sarcoma, and normal adult liver.

Material

Antiserums: Rabbit antihuman-melanoma serum.—Twenty mg. (wet weight) of homogenized human melanoma tissue, suspended in 2 cc. of normal saline was injected into rabbits intravenously 3 times weekly for 3 weeks. Between injections, the tissue homogenate was stored at −30° C. The animals were bled 1 week after the last injection. *Rabbit antihuman-thyroid serum.*—Two hundred mg. of wet thyroid tissue was homogenized in 1 ml. of water and 1 ml. of Freund's adjuvant (*6*). Rabbits received 0.1 ml. portions of this suspension in 3 subcutaneous and 2 intramuscular sites at 2-month intervals for a series of 3 courses. At weekly intervals during this period, the rabbits were also given a total of 12 intraperitoneal injections of whole thyroid saline homogenate—200 mg. wet weight in 12 ml. saline. The rabbits were bled 1 week after the last inoculation. *Rabbit antihuman-globulin serum.*—Three hundred thirty mg. of human globulin (E. R. Squibb & Sons, lot #262–2 poliomyelitis immune globulin) in 2 ml. of solution was homogenized with 2 ml. of Freund's adjuvant (*6*). Three subcutaneous and 2 intramuscular injections of 0.1 ml. portions were given rabbits at monthly intervals for a series of 3 courses. The rabbits also received 4 intravenous injections, 50 mg. each, of the polio-immune globulin during this period. They were bled 1 week after the last intravenous injection.

Test cells: The HeLa cells used in these studies were developed from a single cell of stock HeLa cultures, approximately 4 months prior to the present experiments (*7*). *Strain Raos* was established from a biopsy specimen (obtained at Roswell Park Memorial Institute) of an osteogenic sarcoma, which was growing at the lower end of the femur in a 16-year-old male (R.A.). Histologic sections of the osteogenic sarcoma showed that it was composed of many multinucleated tumor giant cells, malignant sarcoma and osteoblast cells, and poorly formed cartilaginous and osseous elements. A more detailed report of this strain, which is now 1 year old,

will be given at a later time. *Fetal liver cells, strain 407,* were obtained from Dr. F. Deinhardt and Dr. G. Henle, of the University of Pennsylvania. A history of this strain has been reported (*8*). *Permanent strains of rabbit cells RBM-I and RT-I* are fibroblast-like cells which were developed at this institute and derived from the bone marrow and testes of adult rabbits.

Raos cells were grown in a medium containing 10 percent calf serum. The other cell lines were grown in media containing 30 percent calf serum.

Results

The cytotoxic effect of the antiserums was observed on cells grown in Carrel D-3.5 flasks after the medium was replaced by a mixture of 1 part test serum, 4 parts medium 199, and 1 part freshly reconstituted lyophilized guinea-pig serum.

Fifteen to 20 minutes after the addition of rabbit antihuman-melanoma or antihuman-thyroid serum marked cytotoxic changes were observed in HeLa, Raos, and 407-liver cells but not in rabbit cells RBM-I and RT-I. In the absence of complement, heated serum produced no observable changes. When rabbit antihuman-globulin serum or normal pooled rabbit serum was added to cultures in the same dilution as that used for the antihuman-tissue serum, no cellular changes were seen over a 2-hour observation period (table 1).

For the demonstration of reactions with cells by the fluorescein-labeled antibody technique, HeLa, Raos, and 407-liver cells were explanted in slide-cap cultures (*9*). After 24 to 48 hours *in vitro*, the shell vials covering these cultures were removed. The microslides with the firmly adherent cells were washed in Hanks' solution and then fixed in acetone at 5° C. for 30 minutes to 2 hours. The slides were washed under running tap water, rinsed in distilled water, treated with antiserum, and subsequently stained with fluorescein-labeled, horse antirabbit-γ-globulin serum according to the procedures described by Hiramoto and Pressman (*10*). The serums had been absorbed with rat liver sediment to remove nonspecific staining components. Tissue sections were similarly stained according to described methods but without acetone fixation. Tetramethylrhodamine (*11*) was also used as a fluorescent label and gave results identical to those obtained with fluorescein.

TABLE 1.—*Cytotoxic effect of antiserums prepared against human thyroid and human melanoma tissue* *

Cell strains	Rabbit antiserum			Pooled normal rabbit serum
	Antihuman-melanoma	Antihuman-thyroid	Antihuman-globulin	
HeLa	+	+	−	−
Raos	+	+	−	−
407-Liver	+	+	−	−
Rabbit-RBM-I	−	−	−	−
Rabbit-RT-I	−	−	−	−

* + = Cytotoxic; − = noncytotoxic.

When tissue-culture cells were studied, fluorescent staining was observed in all cells and appeared to be fairly uniformly distributed in the cytoplasm. In cells that were not growing in closely packed sheets, the staining was localized around the nuclei. This increase in perinuclear staining was probably due to the cold acetone fixation, which caused an accumulation of proteins at these sites. Cold-acetone-fixed cells stained with hematoxylin and eosin had much of the eosinophilic staining material localized in the same way around the nucleus, with only faint staining of the cytoplasm at the periphery of the cells. In addition to fluorescence in the cytoplasm, some of the nucleoli in both HeLa and Raos cells were stained (*note* figs. 2 and 4).

The staining with labeled antibody could be removed by treating the serums with human liver sediment. When absorptions were carried out with rat liver sediment, the antibody against human stromal elements was not removed and good staining was observed in all the tissue-culture strains. Fixed cells exposed to pooled normal rabbit serum did not exhibit any staining.

Fixed HeLa cells exposed to antihuman-globulin serum, as a control, were unstained except for a few cells that showed brilliant fluorescence of spherical granules of varying sizes in the cytoplasm. This fixation of the rabbit antibody to human globulin in a small number of cells may have been due to cross reaction with bovine globulin, which entered cells from the culture medium by pinocytosis [3] (*12*, *13*) and was present in a stainable form.

The following tissues were also stained and examined: cervical carcinoma, normal human adult liver, and an osteogenic sarcoma. The osteogenic sarcoma and the cervical carcinoma showed weak staining in the cytoplasm of the tumor cells, while the connective-tissue areas showed much more intense staining. (The cytoplasms of normal cervical epithelial cells were also stained.) The adult liver, on the other hand, fixed the antibody only in the sinusoids and connective tissue, while the hepatic cells were unstained. Several other normal tissues (thyroid, kidney, and adrenals) were stained in the connective tissues also. Sections of these tissues treated with normal rabbit serum were not stained.

Treatment of either antiserum with human liver sediment removed all antibodies responsible for staining of cytoplasm or connective tissue.

Discussion

Previous observations indicated that there are similar antigens in cell strains HeLa, 407-liver, and in human connective tissues of biopsy specimens (*4*). In the present studies, antiserums that were prepared in rabbits against a human melanoma and against normal human thyroid reacted in the same manner with the tissue-culture cells noted. A newly established cell line, Raos, which was derived from a biopsy of an osteogenic sarcoma, and surgical specimens of normal and malignant tissues also

[3] Similar staining of cells due to the presence of globulin in nutrient medium has been observed by Dr. Barbara Watson, personal communication (*12*). Cross reaction of antihuman-γ-globulin serum with bovine globulin was reported by Maurer (*13*).

reacted with these antiserums. It is interesting that the 3 tissue-culture lines stain alike, even though Raos has a different morphology as well as origin.

There is the possibility that all the strains were derived from connective-tissue elements. This hypothesis does not seem probable because there are marked differences in morphology between the "epithelial" strains and the Raos strain; in addition, there are differences in histochemical staining reactions, growth rates, and nutritional requirements between the similar epithelium-like HeLa and liver cells, on the one hand, and the fibroblast-like Raos strain, on the other.

One would expect that fluorescein-labeled antibodies which stain tissue-culture cells would also stain the tissue from which these cells were derived. This occurred in *a*) HeLa and cervical carcinoma or normal cervical epithelial cells and *b*) Raos and osteogenic-sarcoma cells when the cytoplasm in these cells was stained. On the other hand, strain 407-liver cells were stained while parenchymal cells of normal human liver were not. Staining did occur in the supporting connective tissue of the liver.

The lack of staining in parenchymal cells of liver by the antihuman-tissue serums used here may have been due to the following possibilities: *a*) Liver strain 407 is not actually derived from liver cells. *b*) Some substances are in cultured liver cells derived from the liver of a 2-month fetus which are different from those in the adult liver cells. *c*) The cells during culture preferentially build up high concentrations of certain antigens—especially those formed in supporting connective tissue.

The fact that liver is stained only in connective-tissue components and not in the parenchymal cells becomes very important when coupled with the observation that human liver sediment removes all antibody capable of staining the cytoplasm of cells of the cervical carcinoma, normal cervix, and osteogenic sarcoma, and all the tissue-culture cells. The antigens, in common, thus appear to be related to stromal elements in the liver.

Coriell *et al.* (*14*) and Rothblat and Morton (*15*) have hypothesized that the presence of pleuropneumonia-like organisms or viral agents may be one cause for the presence of common antigens in permanent cell strains.

Since fresh normal and malignant human biopsy specimens were used, in the work reported here, to prepare antibodies in rabbits, and these reacted with tissue cultures of human cells, the possible presence of pleuropneumonia-like organisms cannot be considered as playing a role in the cross reactivity of the antibodies used in our investigations.

Other results of ours do not correlate with reports of Coriell *et al.* For example, they reported that a long-term culture of rabbit kidney cells shared common antigens with human cell lines as demonstrated by complement-fixation reactions with antiserums prepared against human and monkey cell lines. We have found no such cross reaction with permanent strains of rabbit cells developed in our laboratories. No

"cytotoxic" effects occurred with the serums against human thyroid and melanoma studied here nor were there any cytotoxic effects with serums prepared against tissue-cultured human cells described previously (*3*).

Often there is much uncertainty as to the origin of cells growing in tissue culture. When fragments of normal or malignant tissues or trypsinized cell suspensions are explanted *in vitro*, it is difficult to determine the cells that finally proliferate and give rise to permanent cell lines. If we could identify the cells that are able to grow in tissue culture under certain specific environmental conditions, it would add to our knowledge of the factors that influence the survival and growth of cells *in vitro*. The application of techniques which permit identification of the components of a cell can be very important in determining the histologic origin of cell strains. The studies, in which fluorescent antibodies were used, raise the possibility that the 407-liver cell line, although epithelium-like in morphology, may actually have arisen from connective tissue.

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PLATE

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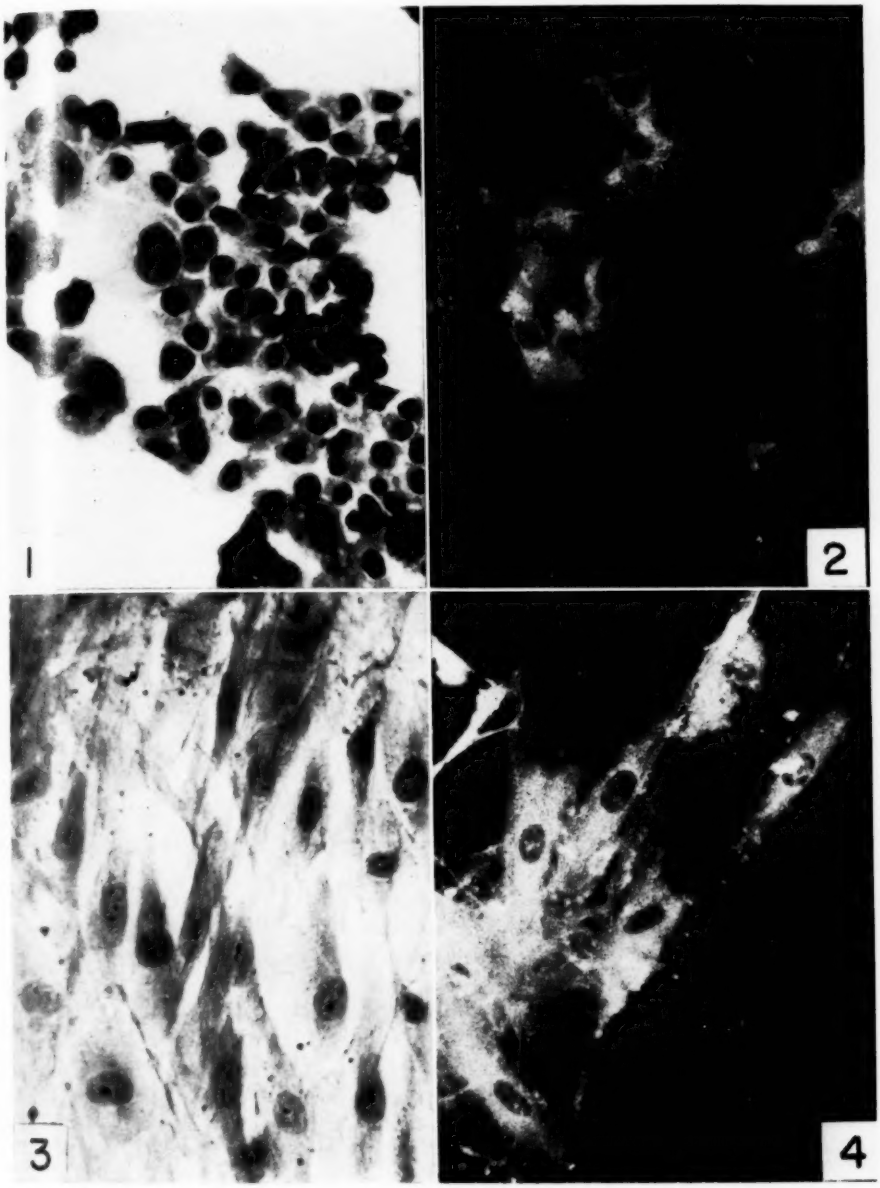
FIGURE 1.—Cells, established from single HeLa cell, which were used for fluorescence and cytotoxicity studies. Carnoy's fixative. Hematoxylin and eosin. × 150

FIGURE 2.—HeLa culture treated with rabbit antihuman-melanoma serum and then stained with fluorescein-labeled, horse antirabbit-γ-globulin. Cold acetone fixation. × 200

FIGURE 3.—Strain Raos. Cells established from biopsy of an osteogenic sarcoma. *Note* marked difference in morphology between these cells and HeLa cells. Carnoy's fixative. Hematoxylin and eosin. × 200

FIGURE 4.—Raos cells treated with rabbit antihuman-melanoma serum and then stained with fluorescein-labeled, horse antirabbit-γ-globulin. Cold acetone fixation. × 200



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Effect of Concentration of Oxygen on Occurrence of Pulmonary Tumors in Strain A Mice [1]

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Summary

The exposure of strain A mice to approximately 100 percent oxygen for 48 hours following the injection of dibenz[*a*,*h*]anthracene resulted in an increase in the number of induced pulmonary tumors, in confirmation of results published earlier. In addition, it was demonstrated that exposure of the mice to the high oxygen concentration for 48 hours, beginning 48 hours after the injection of the carcinogen, likewise increased the occurrence of tumors, but no increase in induced tumors occurred from exposure to the high oxygen concentration either immediately or 48 hours prior to the injection of the carcinogen. The exposure of strain A mice to 8 percent oxygen for 48 hours following the injection of dibenz[*a*,*h*]anthracene resulted in a decrease in the number of tumors induced with the carcinogen. Exposure of newborn strain A mice to approximately 100 percent oxygen for 48 hours did not alter the incidence of spontaneous pulmonary tumors.—**J. Nat. Cancer Inst. 22: 707–717, 1959.**

An increase in induced pulmonary tumors associated with exposure to a high oxygen concentration has been demonstrated in strain A mice in a preliminary study published in 1956 (*1*). These introductory positive results raised many questions regarding the relationship of oxygen tension to the malignant process and opened the way to further study of the general problem.

Although the preliminary study had constituted a series of experiments with one confirming another, it was desirable to repeat the study with the same carcinogen, dibenz[*a*,*h*]anthracene, and the same exposure to oxygen, but with an additional set of controls, injected with the carcinogen and placed in an air chamber similar to the oxygen chamber, to insure better control of such factors as temperature and humidity. This experiment will be described in detail; the results confirm those of the earlier experiment in showing that the high concentration of oxygen did increase the number of pulmonary tumors induced with dibenz[*a*,*h*]anthracene.

[1] Received for publication November 6, 1958.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Following this confirmation, some investigation into the time relationship of the injection of the carcinogen to the exposure to high oxygen seemed appropriate. This would give an indication of whether the carcinogen needed to be present at the time of exposure to high oxygen, or whether the high oxygen could alter the epithelial cells in some way to make them more susceptible to a later exposure to the carcinogen. It was also desirable to ascertain whether or not a decrease in concentration of oxygen below that in air would result in a decrease in the number of induced pulmonary tumors. Another point to be ascertained was whether or not such an increase in pulmonary tumors could be effected by an increase in oxygen tension in mice that were not injected with a carcinogen. These studies were carried out and their results are reported herein.

Effect of Dibenz[*a*,*h*]anthracene Plus High Concentration of Oxygen

Throughout these studies we have continued to use strain A mice that are genetically highly susceptible to pulmonary tumors. The present study constituted 24 series, each consisting of 4 mice that were injected with the carcinogen and then exposed to the high concentration of oxygen; 4 that were injected with the carcinogen and placed in an air chamber similar to the oxygen chamber; and 4 that were injected with the carcinogen and kept in the animal room. Usually 2 series of mice were exposed each week, one from Monday to Wednesday and the other from Wednesday to Friday. Both males and females were used but were segregated in separate series. Litter mates were distributed among the 3 groups in each series. The animals were approximately 2 months of age when they were injected and exposed.

As in the preceding study, dibenz[*a*,*h*]anthracene was used as the carcinogen. By means of a colloidal mill, a dispersion was prepared with 1 mg. dibenz[*a*,*h*]anthracene crystals to 1 cc. water with 0.01 percent aerosol, 2 percent Methocel® 4000, and NaOH to *p*H 8. In order to minimize variation between the series, the dispersion was thoroughly mixed by shaking and was poured into 30-cc. vials, with approximately 25 cc. in each vial. The vials were then sealed. This dispersion was used in all experiments reported herein. In each series, the animals that were to receive an injection were chosen alternately from each of the 3 groups, and new vials were used for each series. The greater uniformity in response of the series of mice in this study as compared with the variation between the series in the earlier study justified this procedure.

Each animal received an injection, in the lateral tail vein, of 0.1 cc. of the dispersion containing 0.1 mg. dibenz[*a*,*h*]anthracene. The mice of each series received an injection about 10:30 a.m., and approximately 30 minutes later the 4 to be exposed to the high concentration of oxygen were sealed in the exposure chamber and the 4 controls were sealed in the air chamber. The 4 room-control mice were returned to the animal room.

For exposure of the animals to the desired gas atmosphere, a flow line was constructed which contained a flow relay, flow meter, and an airtight exposure chamber. Gas supplied from high-pressure cylinders through a

two-stage pressure-reducing valve passed sequentially through a flow relay, flow meter, and then through the chamber. Copper tubing, one-fourth inch in diameter, completed the flow line except for a small segment of rubber tubing connecting the chamber into the flow line. The flow relay and flow meter gave a continuously regulated and monitored flow of 500 cc. per minute ± 1 cc.

The exposure chambers were constructed by fitting a one-eighth inch thick, solid brass lid, seated on a rubber gasket, to the plastic bottom of the usual mouse cage. Adjustable metal bands, attached at their ends to the opposite edges of the brass lid, cradled the cage bottom. Tightening of the metal bands bound the cage bottom firmly against the brass lid and rubber gasket thus creating an airtight seal.

Food and water were available to the animals at all times. The food hopper was suspended from the underside of the lid by means of screws. The usual water-jar assembly was mounted on a #10 rubber stopper; the glass snout of the water jar was forced into a slightly undersized hole drilled in the center of the stopper, which afforded a tight seal around the glass snout as well as a firm mount for the water jar. The whole assembly was mounted on the lid of the chamber by firmly seating the body of the rubber stopper in a machined hole in the lid, thus preserving the air seal. The air inlet and outlet of the cage were constructed from suitable drilled brass rods, which were sealed into the brass lid at opposite ends of the long axis of the lid to afford the longest air path. They were made sufficiently long to extend 3 inches below the under surface of the lid as an aid in obtaining adequate air mixing in the cage.

After 48 hours in the exposure chamber the mice were removed and returned to the animal room. There they were kept in plastic cages (7″ × 11″ × 5″ high) with 8 mice to the cage. Throughout the experiment they were given an unlimited supply of tap water and NCI pellets.[3]

The animals of each series were killed and examined for tumors 4 months after the injection and exposure, with the exception of those of 2 series which were killed and examined after 3 months. Approximately 1 cc. of Fekete's modification of Tellyesniczky's fixative (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) was injected into the lungs intratracheally before they were removed from the chest cavity. After the lungs were removed, the lobes were separated; the tumors appearing on the surface of each lobe were counted with the aid of the dissecting microscope. At least 1 lung tumor from each animal was sectioned and stained with hematoxylin and eosin for histologic examination.

Dosage of carcinogen and technique of injection, age of animals at the start of the experiment, method of setting up littermate controls, care of animals in the animal room, and autopsy and tissue fixation procedures

[3] The National Cancer Institute pellets, formerly referred to as Derwood pellets, are prepared by Herbert Bryant, Inc., Gaithersburg, Md., and are composed of the following: skim-milk powder (human grade), 23.29%; ground whole hard wheat, 65.1%; dried debittered brewer's yeast, 4%; corn oil, 5.8%; sodium chloride (iodinated), 1.4%; iron citrate, U.S.P. #7 powder, 0.13%; shark-liver oil (4.8 million units vitamin A per lb.), 0.2%; and Delstrol® (900,000 units vitamin D per lb.), 0.08%.

described for this experiment were uniform throughout all the experiments reported herein.

Results

The data presented in table 1 clearly confirm the earlier observation that exposure of strain A mice to high concentration of oxygen immediately after being injected with dibenz[*a*,*h*]anthracene significantly increases the number of pulmonary tumors over that induced by the carcinogen alone. Since results for the males did not differ significantly from those for the females, the data were combined in calculating the significance of differences between groups. The average number of pulmonary tumors in the 85 mice exposed to the high concentration of oxygen and killed 4 months later was 14.29, which was significantly greater than the average of 10.56 tumors in the 86 room controls and also significantly greater than the average of 9.93 tumors in the 88 air-chamber controls. The average number of tumors in the air-chamber controls was less than that in the room controls but this difference was not statistically significant. Similar results were noted in the 2 series of mice killed 3 months after the injection and exposure.

TABLE 1.—*Average number of pulmonary tumors in strain A mice 4 months after injection of dibenz*[a,h]*anthracene, with and without subsequent exposure to approximately 100 percent oxygen* *

Series	Sex	O_2 Chamber	Air-chamber control	Room control
1	♂	18.25 (4)	12.25 (4)	12.67 (3)
2	♂	16.75 (4)	7.75 (4)	10.5 (4)
3	♀	14.5 (4)	9.0 (4)	15.5 (4)
4	♀	12.67 (3)	8.0 (4)	11.0 (4)
5	♂	11.25 (4)	11.75 (4)	8.0 (4)
6	♂	11.0 (4)	7.75 (4)	12.25 (4)
7	♀	17.5 (4)	9.75 (4)	8.0 (4)
8	♀	14.75 (4)	7.25 (4)	10.0 (4)
9	♂	15.0 (4)	9.25 (4)	11.0 (4)
10	♂	11.25 (4)	10.5 (4)	6.0 (4)
11	♂	10.5 (4)	9.75 (4)	6.25 (4)
12	♂	14.75 (4)	8.75 (4)	13.67 (3)
13	♀	13.25 (4)	11.0 (4)	14.0 (4)
14	♀	15.0 (2)	10.75 (4)	10.5 (4)
15	♂	20.25 (4)	8.25 (4)	12.5 (4)
16	♂	14.5 (4)	12.25 (4)	9.75 (4)
17	♀	16.0 (4)	9.25 (4)	14.0 (4)
18	♀	14.5 (4)	13.25 (4)	9.75 (4)
19	♂	16.25 (4)	8.0 (4)	9.75 (4)
20	♂	12.25 (4)	11.25 (4)	8.25 (4)
21	♀	14.0 (4)	11.75 (4)	10.25 (4)
22	♀	12.25 (4)	11.0 (4)	10.0 (4)
Total	♂ & ♀	14.29 (85)	9.93 (88)	10.56 (86)
σ		± 5.53	± 3.26	± 3.94
		$P<.01$	$P<.01$	$.2<P<.3$
23†	♀	12.6 (3)	8.0 (3)	6.0 (3)
24†	♀	11.5 (4)	7.25 (4)	9.5 (4)

*Numbers in parentheses indicate number of animals.
†Killed and examined at 3 months following the injection and exposure.

Effect of Varying the Time Relationship Between Injection of Carcinogen and Exposure to Oxygen

This experiment consisted of 4 groups of mice. Mice of the first group were injected with dibenz[*a*,*h*]anthracene, as in the previous experiment, and 48 hours later were placed in the chamber and exposed to approximately 100 percent oxygen for 48 hours. Those of the second group were exposed to the high concentration of oxygen for 48 hours and on removal from the exposure chamber were injected with dibenz[*a*,*h*]anthracene. Those of the third group were exposed to the high oxygen for 48 hours and 48 hours after removal from the chamber were injected with dibenz[*a*,*h*]-anthracene. The mice of the fourth group were injected with dibenz[*a*,*h*]-anthracene and kept as controls. The method of exposure was similar to that of the other experiments except that the chamber used in this experiment held 8 mice. Thus, each series had 8 mice in each group.

Following the exposure and injection, the mice were kept in the animal room for 4 months; at the end of this time they were killed and examined for tumors.

Results

The results presented in table 2 show that the high concentration of oxygen significantly increased the occurrence of pulmonary tumors when the exposure followed the injection of dibenz[*a*,*h*]anthracene. In all series except one, the average number of pulmonary tumors in the group exposed to high oxygen 48 hours after the injection of the carcinogen was greater than the average number in any of the other 3 groups. The overall average number of tumors in the group exposed after the injection

TABLE 2.—*Average number of pulmonary tumors in strain A mice 4 months after injection of dibenz*[a,h]*anthracene and exposure to approximately 100 percent oxygen, with various time intervals**

Series	Sex	DBA + O_2 48 hours later	O_2 + DBA on removal from chamber	O_2 + DBA 48 hours after removal from chamber	DBA controls
1	♀	15.6 (8)	12.4 (7)	10.6 (8)	11.4 (7)
2	♀	18.4 (5)	11.8 (6)	14.0 (6)	12.0 (6)
3	♂	15.8 (8)	10.3 (8)	12.3 (8)	11.8 (8)
4	♀	15.3 (7)	11.3 (8)	11.9 (8)	12.3 (8)
5	♂	10.7 (7)	11.1 (8)	12.1 (8)	13.0 (8)
6	♀	13.0 (7)	10.7 (7)	12.2 (6)	12.1 (8)
7	♂	17.7 (8)	13.9 (8)	14.0 (8)	15.6 (8)
8	♀	14.3 (8)	11.5 (8)	12.4 (7)	9.1 (8)
Total	♂ & ♀	15.0 (58)	11.6 (60)	12.4 (59)	12.2 (61)
σ		±5.36	±5.11	±4.73	±3.93
		$P<.01$	$P<.01$	$P<.01$	$.4<P<.5$

*DBA = Dibenz[*a*,*h*]anthracene. Numbers in parentheses indicate the number of animals.

was statistically significantly greater than that of either group injected after exposure or than that of the controls.

In the groups of mice into which the carcinogen was injected following the exposure to high oxygen on removal from the oxygen chamber or 48 hours after removal, the average number of tumors was not significantly altered from that of the controls which received an injection of the carcinogen but were not exposed to high oxygen.

These results suggest that the increase in pulmonary tumors associated with the increased oxygen occurs when the high oxygen and the carcinogen are concurrently adjacent to the epithelial cells lining the alveoli and from which the tumors arise. At the time of exposure 48 hours after the injection of the carcinogen, some of the crystals were remaining in the capillaries of the lung. There is no evidence that the high oxygen can render the epithelial cells more susceptible to a later exposure to the carcinogen.

In addition to the pulmonary tumors, one of the males injected with dibenz[*a*,*h*]anthracene at the time of removal from the oxygen chamber had, at the time of autopsy, a myoepithelioma in the region of the salivary gland.

Effect of Low Concentration of Oxygen

In this experiment the mice received injections of dibenz[*a*,*h*]anthracene as in the preceding experiments, and were then exposed for 48 hours to an atmosphere containing 8 percent oxygen.[4]

Originally it was planned to have 2 groups of controls as in the first experiment—1 group to be placed in a chamber like the low-oxygen chamber but through which air flowed, *i.e.*, the air-chamber controls, and 1 group to be maintained in the animal room, *i.e.*, the room controls. During the exposure of the first series, however, it was noted that the mice did not eat or drink while in the low-oxygen chamber. This necessitated a third group of controls that were kept in the animal room but were not given food or water during the 48 hours that the low-oxygen group was being exposed. This group is designated as food and water controls.

During the exposure of the first series the air connection for the air chamber failed, eliminating that group. All the subsequent series, however, had 4 groups. There was a total of 13 series and there were 8 mice in each group of each series at the beginning of the experiment.

After receiving an injection and being exposed, the animals were kept in the animal room for 4 months at which time they were killed and examined for tumors, as in the other experiments.

Results

Results of this experiment are presented in table 3. The mice that were exposed to the 8 percent oxygen had on the average fewer lung tumors than did those of any of the 3 groups kept in room air. The difference between the low-oxygen group and either the room-air controls or the food and water controls was significant at a level of less than 1 percent.

[4] Mass spectrometric analysis: 8.1% O_2, 91.6% N, 0.3% A.

TABLE 3.—*Average number of pulmonary tumors in strain A mice 4 months after injection of dibenz*[a,h]*anthracene and exposure to approximately 8 percent oxygen**

Series	Sex	8 percent O_2	Air-chamber controls	Room controls	Food and water controls
1	♂	10.6 (8)		11.0 (7)	
2	♀	8.1 (8)	7.9 (7)	9.3 (8)	11.3 (7)
3	♂	10.6 (7)	6.9 (8)	10.6 (8)	9.8 (8)
4	♂	9.5 (8)	9.1 (8)	11.5 (8)	12.0 (8)
5	♀	10.4 (8)	11.5 (8)	13.5 (8)	9.9 (8)
6	♂	10.0 (8)	11.0 (7)	11.8 (8)	15.9 (8)
7	♀	12.2 (6)	14.0 (6)	9.9 (7)	10.6 (8)
8	♂	12.6 (8)	15.0 (8)	15.4 (8)	12.3 (8)
9	♂	12.5 (8)	14.8 (8)	12.0 (8)	15.9 (7)
10	♀	7.9 (8)	11.9 (8)	13.0 (8)	14.3 (8)
11	♂	14.1 (8)	15.6 (8)	14.6 (8)	10.4 (8)
12	♂	9.4 (8)	12.0 (8)	12.9 (8)	13.5 (8)
13	♀	9.5 (8)	11.5 (8)	12.1 (8)	11.0 (8)
Total	♂ & ♀	10.53 (101)	11.76 (92)	12.15 (102)	12.19 (94)
σ		±4.03	±4.79	±4.73	±4.34
		$P = 0.1$	$P < .01$	$P < .01$	

*Numbers in parentheses indicate number of animals.

In each instance (table 1 and table 3) the air-chamber controls had fewer tumors than the room-air controls. Other experiments (*1*) make it clear that an oxygen depletion of 1 to 2 percent will occur in the air chamber, and therefore it must be recognized that the oxygen tension in the air chambers was below that of the room controls. Unfortunately, the level of oxygen in the air-chamber controls is not accurately known, so that the result cannot be considered as indicating a significant difference. It can only be noted that on the average the number of lung tumors in the air-chamber controls is less than that of the room-air controls and that the oxygen tension of the air chamber would be less than that of the room-air controls.

The difference between the low-oxygen group (8%) and the air-chamber controls is significant only at the 10 percent level. The results together indicate that the air-chamber controls occupy a position intermediate between 20.9 percent (room air) and the low-oxygen group. The low-oxygen group was significantly different from the room-air controls in every instance.

It was evident that the fact that the low-oxygen group did not eat or drink during the exposure period did not contribute to their lowered number of tumors because the average number of tumors in the food and water controls was almost identical to that of the room controls.

Thus, just as the high concentration of oxygen was associated with an increased average number of induced pulmonary tumors in the preceding experiments, the low concentration of oxygen was associated with a decreased average number of induced pulmonary tumors below that of the room-air controls in this experiment.

Exposure of Newborn Mice to High Concentration of Oxygen

Isolated pregnant females of our strain A colony were checked for litters each day at about 9 a.m., and newborn litters were selected for exposure to approximately 100 percent oxygen. At about 10 a.m. the mother and her newborn litter were placed in the exposure chamber where they remained for 48 hours. The chamber and conditions for exposure were the same as that described previously. The exposure was well tolerated by the newborn mice, with no more deaths than normally occurred in mice of this age in the colony. After the 48-hour exposure, the mother and her litter were returned to their cage in the animal room.

When the exposed young were 4 weeks of age, they were weaned and individually identified. At the same time, for each exposed animal, a nonexposed strain A mouse of the same age and sex was weaned, identified, and entered into the experiment as a control. Exposed and control animals were kept segregated and were maintained under the conditions described for the earlier experiments until they were a year old. At that time they were killed and examined for tumors as described.

The exposed and control mice were weighed at weaning and at regular intervals thereafter. Exposure of the mice to the high concentration of oxygen immediately after birth had no effect upon the later health of the animals, as reflected in the comparison of their growth curves with those of the controls.

Results

The results given in table 4 failed to reveal any effect of the high concentration of oxygen on the occurrence of spontaneous pulmonary tumors under the conditions of this experiment. Fifty percent of the exposed males and 36 percent of the exposed females had pulmonary tumors as compared with 55 percent of the control males and 41 percent of the control females. These differences between the experimental and control groups are not statistically significant.

There were more females than males in the exposed group but the difference was not statistically significant. The sex distribution in the controls was governed by that of the exposed mice. In this experiment the incidence of tumors was less in the females than in the males, and this

TABLE 4.—*Occurrence of pulmonary tumors in 1-year-old strain A mice that had been exposed to approximately 100 percent oxygen for 48 hours after birth*

Sex	Mice exposed to approximately 100 percent oxygen		Control mice	
	Number with tumors	Number without tumors	Number with tumors	Number without tumors
♂	37	37	38	31
♀	39	70	45	66

difference was significant below the 5 percent level for the exposed groups. The difference between the control males and females, however, was not significant. Significant sex differences in the occurrence of pulmonary tumors have been observed in mice of certain other experiments, but in some cases the males, and in other cases the females, have had more tumors.

In addition to the mice listed in table 4, 10 exposed males and 3 exposed females were autopsied before they were 1 year old, and 2 of the males and none of the females had pulmonary tumors. Three exposed males and 3 exposed females died without being autopsied. Twelve control males and 2 control females were autopsied before 1 year of age, and 6 of the males and 1 of the females had pulmonary tumors. Four control males and 1 control female died without an autopsy. Other neoplasms observed in this experiment included a reticulum-cell neoplasm in an exposed male, a hepatoma in an exposed male, and a myoepithelioma in the region of the salivary gland of an exposed female. The occurrence of these neoplasms is not unusual for strain A mice.

Discussion

It is recognized that strain A mice are genetically highly susceptible to pulmonary tumors. It may well be that, in choosing pulmonary tumors induced in this strain with intravenously administered dibenz[*a*,*h*]-anthracene, we succeeded in selecting the best situation for demonstrating the effect of oxygen concentration on the neoplastic transformation. Crystals of dibenz[*a*,*h*]anthracene administered in the lateral tail vein presumably go directly to the lungs where they become lodged in the lung capillaries surrounding the alveoli, and it is most probable that the ensuing neoplastic response occurs in the alveolar epithelial cells immediately adjacent to the carcinogen. Nevertheless, it is certain that exposing the test animals to an ambient atmosphere of 100 percent oxygen for 48 hours immediately following administration of the dibenz-[*a*,*h*]anthracene will effect a significant increase in occurrence of pulmonary tumors over that seen in control animals maintained in room air (20.9% oxygen).

Further, the present data suggest that the oxygen effect, as measured by the occurrence of pulmonary tumors, is directly related to the oxygen concentration since an increased number of tumors is found with a high oxygen concentration (100%) and a decreased number is found with a reduced oxygen concentration (8%); again, the control incidence being that of animals maintained in room air (20.9%).

The reduced number of tumors in the 8 percent oxygen series does not appear to be related to the self-imposed calorie-water restriction experienced by the animals during the 48-hour exposure period. It can be seen that the average number in the 8 percent oxygen exposure group was significantly lower than that seen in the calorie-water restricted control animals maintained in room air. It should be noted that the average number of tumors in the calorie-water restricted control animals

kept in room air (20.9% oxygen) was essentially identical with that observed in the *ad libitum* fed controls kept in room air. This result suggests that the 48-hour food and water restriction did not alter the oxygen effect expected in the animals maintained at 20.9 percent oxygen. The reduced number in the 8 percent oxygen exposure group can be considered as a real effect related to the reduced oxygen concentration.

A 48-hour exposure of newborn animals to 100 percent oxygen did not provoke a significant change, in the 1-year tumor incidence, from that observed in control animals maintained in room air. This result indicates that the 48-hour exposure to molecular oxygen does not by itself increase the occurrence of lung tumors. To obtain an oxygen effect, it appears that the presence of the carcinogen (dibenz[*a*,*h*]anthracene) is required.

Other experiments conducted during this study tend to confirm this impression. For example, the average number of pulmonary tumors in animals receiving dibenz[*a*,*h*]anthracene immediately following the completion of a 48-hour exposure to 100 percent oxygen was not different from that seen in control animals maintained only in room air. Withholding the dibenz[*a*,*h*]anthracene for 48 hours following the completion of the 100 percent oxygen exposure did not alter the average number of tumors from that seen in the control animals. The average number of pulmonary tumors in the animals of this experimental series was what would be expected in animals maintained in room air (20.9% oxygen). It is obvious that the prior exposure to 100 percent oxygen did not create a "lasting condition," which upon subsequent administration of the dibenz[*a*,*h*]-anthracene led to an increased number of pulmonary tumors. Thus, the effective concentration of oxygen for establishing the pulmonary tumor response appears to be that concentration of oxygen in which the animal is maintained following the administration of the carcinogen. In this regard, it should be noted that exposure to the 100 percent oxygen 48 hours after the administration of the dibenz[*a*,*h*]anthracene gave the expected increase in number of pulmonary tumors.

Smith, in confirming the original observations of Paul Bert on oxygen toxicity, noted that oxygen in increased tensions acted as an irritant and produced inflammatory changes in the lungs of mice (*2*). Other workers have pointed out that the pulmonary changes vary in character and intensity, depending on the tension of the oxygen and duration of the exposure (*3*).

Any discussion as to the effect of an inflammatory reaction on the number of lung tumors arising from the dibenz[*a*,*h*]anthracene exposure must await further experimentation. It is worth noting, however, that the number of lung tumors found after 1 year, in mice that in the absence of dibenz[*a*,*h*]anthracene were exposed immediately after birth to 100 percent oxygen for 48 hours, was not significantly different from the values obtained from the littermate controls maintained at 20.9 percent oxygen.

Any additional discussion on the possible relationship between the effect of oxygen tension on occurrence of pulmonary tumors and its

effect on mutation rate, as mentioned in the previous paper (*1*), should also await further experimentation.

The mechanism by which molecular oxygen alters the number of the dibenz[*a*,*h*]anthracene-induced pulmonary tumors in strain A mice remains obscure. It can be recognized in these experiments that the oxygen effect was obtained only in the presence of the carcinogen or following an exposure to the carcinogen, and that by itself a 100 percent oxygen exposure of 48 hours was apparently unable to alter the occurrence of pulmonary tumors.

The present data would suggest that molecular oxygen could have its effect by creating a physiologic state of relative susceptibility of the alveolar epithelial cells to the action of the dibenz[*a*,*h*]anthracene. The observable degree of susceptibility of the epithelial cells created by any concentration of oxygen, as indicated by the occurrence of pulmonary tumors, is maintained only as long as that concentration of oxygen is maintained in the presence of the carcinogen. The degree of response, *i.e.*, the average number of pulmonary tumors found after exposure to 100, 20.9, and 8 percent, appears to vary directly with the oxygen concentration in the ambient atmosphere in which the animal is maintained following the administration of the dibenz[*a*,*h*]anthracene.

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Statistical Aspects of the Analysis of Data From Retrospective Studies of Disease [1]

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Summary

The role and limitations of retrospective investigations of factors possibly associated with the occurrence of a disease are discussed and their relationship to forward-type studies emphasized. Examples of situations in which misleading associations could arise through the use of inappropriate control groups are presented. The possibility of misleading associations may be minimized by controlling or matching on factors which could produce such associations; the statistical analysis will then be modified. Statistical methodology is presented for analyzing retrospective study data, including chi-square measures of statistical significance of the observed association between the disease and the factor under study, and measures for interpreting the association in terms of an increased relative risk of disease. An extension of the chi-square test to the situation where data are subclassified by factors controlled in the analysis is given. A summary relative risk formula, R, is presented and discussed in connection with the problem of weighting the individual subcategory relative risks according to their importance or their precision. Alternative relative-risk formulas, R_1, R_2, R_3, and R_4, which require the calculation of subcategory-adjusted proportions of the study factor among diseased persons and controls for the computation of relative risks, are discussed. While these latter formulas may be useful in many instances, they may be biased or inconsistent and are not, in fact, averages of the relative risks observed in the separate subcategories. Only the relative-risk formula, R, of those presented, can be viewed as such an average. The relationship of the matched-sample method to the subclassification approach is indicated. The statistical methodology presented is illustrated with examples from a study of women with epidermoid and undifferentiated pulmonary carcinoma.—**J. Nat. Cancer Inst. 22: 719–748, 1959.**

Introduction

A retrospective study of disease occurrence may be defined as one in which the determination of association of a disease with some factor is based on an unusually high or low frequency of that factor among diseased persons. This contrasts with a forward study in which one looks instead

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for an unusually high or low occurrence of the disease among individuals possessing the factor in question. Each approach has its advantages. Among the desirable attributes of the retrospective study is the ability to yield results from presently collectible data, whereas the forward study usually requires future observation of individuals over an extended period (this is not always true; if the status of individuals can be determined as of some past date, the data for a forward study may already be at hand). The retrospective approach is also adapted to the limited resources of an individual investigator and places a premium on the formulation of hypotheses for testing, rather than on facilities for data collection. For especially rare diseases a retrospective study may be the only feasible approach, since the forward study may prove too expensive to consider and the study size required to obtain a respectable number of cases completely unmanageable.

In the absence of important biases in the study setting, the retrospective method could be regarded, according to sound statistical theory, as the study method of choice. This follows from the much reduced sample sizes required by this approach and may be illustrated by the following extreme example. If a disease attack rate of 10 per 100,000 among 50 percent of the population free of some factor were increased tenfold among the other half of the population subject to the factor, a retrospective study of 100 cases and 100 controls would, with high probability, reveal this significantly increased risk. On the other hand, a forward study covering 2,000 persons, half with and half without the factor, would almost certainly fail to detect a significant difference. For comparable ability to find the type of increased risk just indicated, a forward study would need to cover about 500 times as many individuals as the corresponding retrospective study. The disparity in the required number of persons to be studied could, of course, be reduced by lengthening the follow-up period for forward studies to increase the experience in terms of person-years observed. The larger sample size required for the forward study reflects principally the infrequent occurrence of the disease entity under investigation. In the example illustrated, uncovering 100 cases of disease in a forward study would require either 100,000 individuals with the factor or 1,000,000 without. For diseases with a higher probability of occurrence the disparity in required size between retrospective and forward studies would be progressively reduced.

The retrospective study might be looked upon as a natural extension of the practice of physicians since the time of Hippocrates, to take case histories as an aid to diagnosis. Its guise has varied with respect to the means of measuring the prevalence of the suspect factor among diseased persons and the criteria for determining unusual departures from normal experience. When an association is so marked, as in Percival Pott's observations on the representation of chimney sweeps among cases of scrotal cancer, no further quantitative data are required to perceive its significance.

The retrospective approach has often been employed in studies of com-

municable diseases, one illustration being Snow's observations (*1*) on a common water supply for cholera cases in an area served by several sources (there would have been no element of unusualness had there been but one water supply). When a disease is epidemic in a circumscribed locality, the disease-free population in the same area offers a natural contrast. The method may be used successfully for endemic diseases as well. Holmes, in reaching his conclusions on the communicable nature of puerperal fever (*2*), noted particularly that a large number of women with puerperal fever had been attended by the same physicians. In this context it should be emphasized that communicable disease investigations have often combined retrospective and forward study methods. For example, Snow supplemented his retrospective observations on water supply by a contrast of cholera rates among subscribers of the Southwark and Vaûxhall water company with the experience of persons served by the Lambeth water company within the same area.

When a disease occurs sporadically, or its occurrence is not confined to a well-defined group (such as women at childbirth), a choice of controls is not immediately evident. For cancer and other diseases characterized by high fatality rates, a study restricted to decedents might use persons dying from other causes as controls. Rigoni Stern adopted this technique in deducing the relationship of cancer of the breast and of the uterus to pregnancy history (*3*). Some contemporary studies have also used deaths from other causes as controls (*4*, *5*).

The present-day controlled retrospective studies of cancer date from the Lane-Claypon paper on breast cancer published in 1926 (*6*). This report is significant in setting forth procedures for selecting matched hospital controls and relating them to a consideration of study objectives. Retrospective techniques have since been applied in several investigations of cancer, including the following partial list of current references for a few primary sites: bladder (*7–10*), breast (*11–13*), cervix (*13–16*), larynx (*17*, *18*), leukemia (*19*), lung (*18*, *20–27*), and stomach (*13*, *28–30*).

Statisticians have been somewhat reluctant to discuss the analysis of data gathered by retrospective techniques, possibly because their training emphasizes the importance of defining a universe and specifying rules for counting events or drawing samples possessing certain properties. To them, proceeding from "effect to cause," with its consequent lack of specificity of a study population at risk, seems an unnatural approach. Certainly, the retrospective study raises some questions concerning the representative nature of the cases and controls in a given situation which cannot be completely satisfied by internal examination of any single set of data.

Only a few published papers have treated the statistical aspects of retrospective studies. Cornfield discussed the problem in terms of estimated measures of relative and absolute risks arising from contrasts of persons with and without specified characteristics (*31*). His paper was concerned with the simple situation of a homogeneous population of cases and controls, presumably alike in all characteristics except the one under

investigation, which could be represented by a single contingency table. In a later contribution he handled the problem of controlling for other variables by adjusting the distribution of controls to the observed distribution of cases (*16*). Dorn briefly mentions retrospective studies with emphasis on such topics as sources of data, choice of controls, and validity of inferences (*32*).

This paper presents a method for computing relative risks for retrospective study contrasts, which controls for the effects of other variables by use of the basic statistical principle of subclassification of data. The related problem of significance testing is also considered. Since details of statistical treatment are conditioned by study objectives, data collection methods, choice of a control series, and the use of matched or unmatched controls, these topics are also discussed briefly.

Objectives

Retrospective studies are relatively inexpensive and can play a valuable role as scouting forays to uncover leads on hitherto unknown effects, which can then be explored further by other techniques. The effects may be novel and not suggested by existing data, as in the pioneer work on the association of smoking and lung cancer or the association of blood type and gastric cancer, or they may represent refinements of current knowledge. The latter category might include collection of lifetime residence and/or work histories to elaborate differences in incidence and mortality which appear when some diseases are classified by last place of residence or last occupation of the newly diagnosed case or decedent.

With diseases of low incidence the controlled retrospective study may be the only feasible approach. Here emphasis should be placed on assembling results from several studies. Before accepting a finding and offering an interpretation, scientific caution calls for ascertaining whether it can be reproduced by others and in other administrative settings having their own peculiar biases.

A primary goal is to reach the same conclusions in a retrospective study as would have been obtained from a forward study, if one had been done. Even when observations for a forward study have been collected, a supplementary retrospective approach to the same body of material may prove useful in collecting more data on points not covered in the original study design or in amplifying suggestive associations appearing in the initial forward-study results.

The findings of a retrospective study are necessarily in the form of statements about associations between diseases and factors, rather than about cause and effect relationships. This is due to the inability of the retrospective study to distinguish among the possible forms of association—cause and effect, association due to common causes, etc. Similar difficulties of interpretation arise in forward studies as well. A forward study, to avoid these difficulties, would need to be performed with the preciseness of a laboratory experiment. For example, such a study of associations with cigarette smoking would require that an investigator

randomly assign his subjects in advance to the various smoking categories, rather than simply note the categories to which they belong. The inherent practical difficulties of such an enterprise are evident.

In addition to the failings shared with the forward study, the retrospective study is further exposed to misleading associations arising from the circumstances under which test and control subjects are obtained. The retrospective study picks up factors associated with becoming a diseased or a disease-free *subject*, rather than simply factors associated with presence or absence of the disease. The difficulties in this regard may be most pronounced when the study group represents a cross section of patients alive at any time (prevalence), including some who have been ill for a long period. Inclusion of the latter may lead to identification of items associated with the course of the illness, unrelated to increased or decreased risk of developing the disease. The theoretical point has been raised that factors conducive to longer survival of patients may be found in "prevalence" samples and interpreted erroneously as being associated with excess liability to the disease (*33*). Loopholes of this type are minimized when investigations are restricted to samples of newly diagnosed patients (incidence).

A partial remedy for these uncertainties lies in employing a conservative approach to interpretation of the associations observed. Recognizing the ease with which associations may be influenced by extraneous factors, the investigator may require not only that the measure of relative risk be significantly different from unity but also that it be importantly different. He may, for instance, require that the data indicate an increased relative risk for a characteristic of at least 50 percent, on the assumption that an excess of this magnitude would not arise from extraneous factors alone. However, the use of such conservative procedures emphasizes a corresponding need to pinpoint the disease entity under study. A strong relationship between a factor and a disease entity might fail to be revealed, if the entity was included in a larger, less well-defined, disease category. After the event from data now at hand, we know that a study of the association of cigarette smoking with epidermoid and undifferentiated pulmonary carcinoma is more revealing than an inquiry covering all histologic types of lung cancer.

Multiple Comparison Problem

The present-day retrospective study is usually concerned with investigating a variety of associations with a disease, little effort being involved in acquiring, within limits, added information from respondents. The results may be analyzed in a number of ways: the various factors may be investigated separately, without regard to the other factors; they may be investigated in conjunction with each other, a particular conjunction being considered a factor in its own right; or, more commonly, a factor may be tested with control for the presence or absence of other factors. Thus, if the role of cigarette smoking and coffee drinking in a given disease are under study, the possible comparisons include the relative

risk of disease for individuals who both smoke and drink as opposed to all other persons, or as opposed to those who neither smoke, nor drink coffee. In addition, the relative risk associated with smoking might be obtained separately for drinkers and nondrinkers of coffee, with a weighted average of these two relative risks constituting still another item. Conversely, risks associated with coffee drinking, with adjustments for cigarette smoking, could be computed.

The potential comparisons arising from a comprehensive retrospective study can be large. Almost any reasonable level of statistical significance used to test a single contrast, when applied to a long series of contrasts, will, with a high degree of probability, result in some contrasts testing significant, even in the absence of any real associations. The usual prescription for coping with this multiple comparison problem—requiring individual comparisons to test significant at an extreme probability level to reduce the number of associations incorrectly asserted to be true—would result only in making real associations difficult to detect.

However, the multiple comparison problem exists only when inferences are to be drawn from a single set of data. If the purpose of the retrospective study is to uncover leads for fuller investigation, it becomes clear there is no real multiple significance testing problem—a single retrospective study does not yield conclusions, only leads. Also, the problem does not exist when several retrospective and other type studies are at hand, since the inferences will be based on a collation of evidence, the degree of agreement and reproducibility among studies, and their consistency with other types of available evidence, and not on the findings of a single study.

Nevertheless, it would be wise to employ testing procedures which do not lead to a superabundance of potential clues from any one study. This may be achieved by employing nominal significance levels in testing factors of primary interest incorporated into the design of an investigation and applying more stringent significance tests to comparisons of secondary interest or to comparisons suggested by the data. For the usual problem of multiple significance testing, this would be equivalent to allocating a large part of the desired risk of erroneous acceptance of an association as real to a small group of comparisons where fruitful results were anticipated, and parceling out the remainder of the available risk to the large bulk of comparisons of a more secondary nature. This minimizes the risk of diluting, through inclusion of many secondary comparisons, the chances for detecting an important primary effect.

Representative Nature of Data

The fundamental assumption underlying the analysis of retrospective data is that the assembled cases and controls are representative of the universe defined for investigation. This obligates the investigator not only to examine the data which are the end product but also to go behind the scenes and evaluate the forces which have channeled the material to his attention, including such items as local practices of referral to special-

ists and hospitals and the patient's condition and the effect of these items on the probability of diagnosis or hospital admission. We re-emphasize that this requires the exercise of judgment on the potential magnitude of biases and as to whether they could result in factors seeming to be related to a disease, in the absence of a real association of the factor with presence or absence of the disease. The danger of bias may be greatest in working with material from a single diagnostic source or institution.

Among the more important practical considerations affecting retrospective studies is that they are ordinarily designed to follow the line of least resistance in obtaining case and control histories. This means that cases and controls will often be hospital patients rather than persons in the general population outside hospitals. As a result, any factor which increases the probability that a diseased individual will be hospitalized for the disease may mistakenly be found to be associated with the disease. For example, Berkson (*34*) and White (*35*) have pointed out that positive association between two diseases, not present in the general population, may be produced when hospital admissions alone are studied, because persons with a combination of complaints are more likely to require hospital treatment. In theory, bias might also be produced in reverse manner, if the suspect factor diminished the probability of hospitalization for other diagnoses used as controls. The difficulties are not unique for hospital patients. Similar loopholes in interpretation may be advanced for any special groups used as sources of cases and controls.

However, a mere catalogue of biases arising from the possibly unrepresentative nature of a sample of cases and controls should not *ipso facto* invalidate any study findings. This is a substantive issue to be resolved on its merits for a specific investigation. Collateral evidence may provide information on the potential magnitude of bias and the size of spurious associations which could result. In some situations the difference between cases and controls may be so great that postulation of an unreasonably large bias would be required. Whether he consciously recognizes it or not, the investigator must always balance the risks confronting him and decide whether it is more important to detect an effect, when present, or to reject findings, when they may not reflect the true situation. If opportunities for further testing exist, one should not be too hasty in rejecting an association as an artifact arising from the method of data collection, and in foreclosing exploration of a potentially fruitful lead.

Because of the important role retrospective studies play in studies of human genetics, mention may be made of a bias frequently encountered in studies dealing with the familial distribution of diseases. A frequently used procedure takes a group of diagnosed cases for a disease in question and a group of controls and compares the prevalence of this disease among relatives of the probands and controls. The bias arises from the unrepresentative nature of the probands with respect to familial distribution and is known in other fields as "the problem of the index case" or "the effect of method of ascertainment." It has long been recognized that the

characteristics for a random sample of families will differ from those for families to whom the investigator's attention has been directed because the family rosters include individuals selected for study on the basis of a specified attribute. For example, data on family size (number of children) obtained from siblings, rather than parents, are biased, since two or three potential index cases are present in the population for two- and three-child families as opposed to one for one-child families and none for childless couples. The analogy for disease occurrence is apparent. Families with two or three cases of the disease under study may have double or triple the probability of being represented by individuals in source material and having a representative selected as a proband than families with only one case. An appropriate analysis for this situation in studies of family size and birth order has been discussed by Greenwood and Yule (*36*), which takes account of the probability of family representation in proband data. Haenszel (*37*) has applied their correction to gastric-cancer data reported by Videbaek and Mosbech (*38*) and found the correction to reduce the originally reported fourfold excess of gastric cancer among relatives of probands, as compared to relatives of controls, to one of about 60 percent.

One remedy for the weakness of the retrospective approach to problems involving association of diseases and familial distribution would be to place greater reliance on forward observations of defined cohorts for data on these topics.

Controls

While easier accessibility to and lesser expense of hospital controls are important considerations, they should not deter one from collecting control data for a sample representing a more general population, if the latter are demonstrably superior. Some of the uncertainties about the superiority of hospital or general population controls arise from the need to maintain comparability in responses. The dependence of retrospective studies on comparability of responses from cases and controls cannot be overemphasized. When more accurate answers can be obtained from controls in a medical-care environment, the gain in comparability of responses for these controls could outweigh the other advantages to be derived from the more representative nature of general population controls. The difficulties may be illustrated by the experience with smoking histories. Hospital controls invariably yield a higher proportion of smokers for each sex than controls of comparable age drawn from the general population (*27*). Does this mean more complete smoking histories are collected in hospitals or does it imply that smokers have higher hospital admission rates? If the first alternative is correct, hospital controls are the appropriate choice for measuring the association of smoking history with a given disease. The second alternative calls for general population controls and in this situation the use of hospital controls yields underestimates of the degree of association.

Dual hospital and general population controls would have some merit. If control data from the two sources were in agreement, this would rule

out some alternative interpretations of the findings. In the event of disagreement, its extent could be measured and alternate calculations made on the degree of association between an event and a suspect antecedent characteristic. Where the two sets of controls lead to substantially different results, a cautious and conservative interpretation is indicated.

Some topics, such as those bearing on sex practices and use of alcohol, may be amenable to study only within a clinical setting, and the collection of general population data on these items may prove impractical. The limitations of general population controls in this regard may have been overstressed, and empirical trials to test what information can be collected in household surveys should be encouraged instead of dismissing the possibility with no investigation whatsoever. Whelpton and Freedman, for example, have reported some success in collecting histories of contraceptive practices in interviews of a random sample of housewives (*39*).

When hospital controls are chosen, some precautions may be built into the study. Within limitations on the nature of controls imposed by a study hypothesis, controls drawn from a wide variety of diseases or admission diagnoses should be preferred. This permits examination of the distribution of the study characteristics among subgroups to check on internal consistency or variation among controls. This affords protection against two sources of error: *a*) attributing an association to the disease under investigation, when the effect is really linked to the diagnosis from which controls were drawn, and *b*) failure to detect an effect because both the study and control diseases are associated with the suspect factor. The latter is far from impossible. Both tuberculosis and bronchitis have exhibited association with smoking history and the use of one disease or the other as a control could easily lead to missing the association with smoking history. Similarly, patients with coronary artery disease would not constitute suitable controls for a study of the relationship of smoking and bladder cancer and *vice versa*, since the investigator would probably conclude that smoking was not related to either disease, when in truth it appears related to both. When there is definite evidence that two diseases are associated, for example, pernicious anemia and stomach cancer, the use of one as a control for the other is contraindicated, unless the study is specially designed to elucidate some aspects of the relationship.

It is always advantageous to include several items in a questionnaire for which general population data are available. This could be considered a partial substitute for dual hospital and general population controls. Disparity among cases, hospital controls, and general population controls on several general characteristics unrelated to the study hypothesis may be regarded as warning signals of the unrepresentative nature of the hospital cases and controls.

Where possible, interviews should be conducted without knowledge of the identity of cases and controls to guard against interviewer bias, although administrative reasons will often prevent attainment of "blind" interviews. In cooperative studies employing several interviewers, the

magnitude of interviewer bias may be diminished, since it is unlikely that all interviewers will share the same bias in concert. In special circumstances, such as those prevailing at Roswell Park Memorial Institute, admissions may be interviewed before diagnosis, and hence before the identity of cases and controls is established. This feature requires a comprehensive, general purpose interview routinely administered to all admissions, which may restrict its use to publicly supported institutions diagnosing and treating neoplastic diseases or other specialized disease entities. Several epidemiological contributions for specific cancer sites have been based on the unique control data available from Roswell Park Memorial Institute (*9, 11, 12, 30, 40–43*), which are particularly valuable for collation with studies depending on more conventional sources of controls to evaluate interviewer bias and related issues.

Some patients interviewed as diagnosed cases will subsequently have their diagnoses changed. This may be turned to advantage. If scrutiny of the data for the erroneously diagnosed group reveals they had histories resembling those for the control rather than the case series, as Doll and Hill found in their study of smoking and lung cancer (*21*), this would constitute evidence against interviewer bias.

In investigations of a cancer site the association of a factor may often be restricted to a specific histologic type or a well-defined portion of an organ. The finding that epidermoid and undifferentiated pulmonary carcinoma is more strongly related to smoking history than adenocarcinoma of the lung is now well established. The range of explanations for the observed deficit of epidermoid carcinoma of the cervix in Jewish women as compared to other white women is greatly circumscribed by the presence of about equal numbers of adenocarcinoma of the corpus in both groups. When these finer diagnostic details or their significance are unknown to the interviewer, another check on interviewer bias is provided. Furthermore, the confirmation in repeated studies of an association limited to a specific histologic type or a detailed site will lend credence to an etiological interpretation of the association. Repeated confirmation is an essential element. Otherwise, a very specific association may be a reflection of the multiple comparison problem; if enough contrasts are created by fractionation of a single set of data, some apparently significant result is likely to appear. For this reason it would be desirable to reproduce such provocative results as Wynder's finding that use of alcohol was more strongly associated with cancer of the extrinsic larynx than of the intrinsic larynx (*18*), and Billington's report that prepyloric and cardiac neoplasms of the stomach were associated with blood group A and those located in the fundus with blood group O (*44*).

Discussion of matched controls in relation to the analysis and the computation of relative risks is deferred to a later section. One consideration on matched controls arising in the planning and development of a study should be mentioned here. Obviously, if the risk of disease changes with age an apparent association of the disease with other age-related factors may result. Other apparent associations with race, sex,

nativity, etc., may arise in a similar manner. In devising rules for selecting controls, those factors known or strongly suspected to be related to disease occurrence should be taken into account if unbiased and more precise tests of the significance of the factors under investigation are desired. A sensible rule is to match those factors, such as age and sex, the effect of which may be conceded in advance and for which strong evidence is available from other sources, such as mortality data and morbidity surveys. When a factor is matched, however, it is eliminated as an independent study variable; it can be used only as a control on other factors. This suggests caution in the amount of matching attempted. If the effect of a factor is in doubt, the preferable strategy will be not to match but to control it in the statistical analysis. While the logical absurdity of attempting to measure an effect for a factor controlled by matching must be obvious, it is surprising how often investigators must be restrained from attempting this.

When a minimum of matching is involved, the importance of establishing, precisely and in advance, the method by which controls are selected for study increases. The rule should be rigid and unambiguous to avoid creating effects by subconscious selection and manipulation of controls. The problem is similar to that encountered in therapeutic trials where a protocol spelling out all the contingencies and actions to be taken in advance is, along with random assignment of cases and controls, the major bulwark against bias.

To reduce interview time and expense there are advantages in procedures for selecting controls which permit a case and the corresponding controls to be interviewed in a single session, particularly if travel to several institutions is involved. In practice, this favors selecting controls from a hospital patient census rather than from hospital admission lists. The difficulty with hospital admissions is that there is no guarantee that the controls will be available in the hospital at the time the diagnosed case is interviewed. This point seems more important than the fact that patients with diagnoses requiring long-term stays are overrepresented in a current hospital census (*45*). If the latter is an important issue, it may be handled in analysis through subclassification of controls by diagnosis.

Normally there will be little difficulty in reconciling these considerations into a harmonious set of rules. The items to be matched often lend themselves to a procedure for specifying controls. In a recent study on female lung cancer we found that the definition of two controls as the next older and the next younger women in the same hospital service, present on the day the case was interviewed, met the requirements just outlined (*27*). The controls were uniquely defined, the records establishing their identity were readily available on the service floor, interviews could be completed in one day, and a provision for balancing ages of cases and controls was incorporated. Simultaneous interviews of cases and controls may be more than an administrative convenience. If the prevalence of the associated factor is rapidly shifting over time,

failure to control time of interview could obscure or exaggerate an association.

Some Statistical Tools

To progress further, questions on the representative nature of the case and control series must have been resolved affirmatively. With this condition in mind, let us suppose that a controlled retrospective study has been conducted and that the number of diseased cases, N_1, consists of A individuals with the factor being investigated and B free of the factor, while the number of controls, N_2, consists of C individuals with, and D individuals without the factor. Let $M_1 = A + C$, $M_2 = B + D$, $T = N_1 + N_2 = M_1 + M_2 = A + B + C + D$. What statistical evidence is there for the presence of an association and what is an appropriate measure of the strength of the association?

A commonly employed statistical test of association is the chi-square test on the difference between the cases and controls in the proportion of individuals having the factor under test. A corrected chi square may be calculated routinely as

$$(|AD-BC|-\tfrac{1}{2}T)^2T/N_1M_1N_2M_2$$

and tested as a chi square with 1 degree of freedom in the usual manner.

A suggested measure of the strength of the association of the disease with the factor is the apparent risk of the disease for those with the factor, relative to the risk for those without the factor. Consider that a population falls into the four possible categories and in the proportions indicated by the following table:

	With factor	Free of factor	Total
With disease	P_1	P_2	$P_1 + P_2$
Free of disease	P_3	P_4	$P_3 + P_4$
Total	$P_1 + P_3$	$P_2 + P_4$	1

The proportion of persons with the factor having the disease is $P_1/(P_1 + P_3)$, while the corresponding proportion for those free of the factor is $P_2/(P_2 + P_4)$. Relatively then, the risk of the disease for those with the factor is $P_1(P_2 + P_4)/P_2(P_1 + P_3)$. On a sampling basis this quantity may be estimated either by drawing a sample of the general population and estimating P_1, P_2, P_3, and P_4 therefrom or estimating $P_1/(P_1 + P_3)$ and $P_2/(P_2 + P_4)$ separately from samples of persons with, and persons free of, the factor.

It may be noted, however, that if the relative risk as defined equals unity, then the quantity P_1P_4/P_2P_3 will also equal unity. Further, for diseases of low incidence where the values for P_1 and P_2 are small in comparison with P_3 and P_4 it follows, as has been pointed out by Cornfield (*31*), that P_1P_4/P_2P_3 is also a close approximation to the relative risk. This latter approximate relative risk can properly be estimated from the two sample approaches described or from samples drawn on a retrospective basis; that is, separate samples of persons with, and persons free of, the disease. The sample proportions of persons with, and free

of, the factor in the retrospective approach provide estimates of $P_1/(P_1 + P_2)$ and of $P_2/(P_1 + P_2)$ from the sample having the disease and of $P_3/(P_3 + P_4)$ and of $P_4/(P_3 + P_4)$ from the disease-free sample. The estimate of P_1P_4/P_2P_3 is obtained by appropriate multiplication and division of these four quantities.

Whichever of the three methods of sampling is employed, the estimate of the approximate relative risk, P_1P_4/P_2P_3, reduces simply to AD/BC, where A, B, C, and D are defined in the manner stated in the first paragraph of this section. Also, the chi-square test of association given, which is essentially a test of whether or not the relative risk is unity, is equally applicable to all three sampling methods.

In the foregoing the two basic statistical tools of the epidemiologist for retrospective studies, the chi-square significance test and the measure of a relative risk, have been described for a relatively simple situation, one in which to all intents there is a single homogeneous population. The more complex situations confronting the epidemiologist in actual practice and the corresponding modifications in the statistical procedures will be presented.

Two other statistical problems may be noted here. One is the determination of how large a retrospective study to conduct. This depends on how sure we wish to be that the study will yield clear evidence that the relative risk is not unity, when it in fact differs from unity to some important degree. Application of this statistical technique requires reinterpreting a relative risk greater than unity into the corresponding difference between the diseased and the disease-free groups in the proportion of persons with the factor. For example, suppose an attack rate of 20 percent, given a normal rate of 10 percent, is worth uncovering. Suppose further that the factor associated with the increased disease rate affects 20 percent of the population. The population would then be distributed as follows:

	With factor	Free of factor	Total
With disease	P_1=4%	P_2=8%	12%
Free of disease	P_3=16%	P_4=72%	88%
Total	20%	80%	100%

The required retrospective study should be large enough to differentiate between a 33.3 percent $[P_1/(P_1 + P_2)]$ relative frequency of the factor among diseased individuals and an 18.2 percent $[P_3/(P_3+P_4)]$ relative frequency among disease-free individuals. The usual procedures for determining required sample sizes to differentiate between two binomial proportions are applicable in this situation.

While rigorous extension of this procedure to the more complex situations to be considered is not too simple, it can readily be adapted to secure approximations of the necessary study size. One might, for example, start by estimating the over-all required sample size following the procedure just indicated for differentiating between two sample proportions, assuming that cases and controls are homogeneous with

respect to factors other than the one under investigation. Suppose on an over-all basis it is determined that the study should include $N_1 = 200$ disease cases and $N_2 = 200$ controls, but that the study data will be subclassified for purposes of analysis. Ignoring mathematical complications resulting from variations in binomial parameter values within individual subclassifications, we may interpret the above values of N_1 and N_2 as roughly meaning that the total information required for the study is $N_1N_2/(N_1 + N_2) = 100$. The objective should then be to assign values to N_{1i} and N_{2i} to obtain a total score of 100 for the cumulated information over all the subclassifications, $\Sigma N_{1i}N_{2i}/(N_{1i} + N_{2i})$, where N_{1i} and N_{2i} are the number of cases and controls in the *ith* subclassification.

This formulation of required total information brings out some aspects of retrospective study planning which are considered later in this paper. For instance, if any N_{1i} or N_{2i} is zero, no information is available from that particular category. Much of the benefit of a large N_{1i} (or N_{2i}) in any particular category is lost if the corresponding N_{2i} (or N_{1i}) is small. It is normally desirable to have N_{1i} and N_{2i} values commensurate with each other; for fixed totals, ΣN_{1i} and ΣN_{2i}, the total information in an investigation will be at a maximum if the degree of crossmatching is equal in all subclassifications with a constant case-control ratio of $\Sigma N_{1i}/\Sigma N_{2i}$. Maintaining a fixed case-control ratio among categories need not preclude assigning more cases and controls to specific categories. Larger numbers may be desired for categories of crucial interest to the study or for categories which represent greater segments of the population.

The information formula also reveals the limits for adjusting the relative numbers of diseased and control cases. It shows that if the number of controls (N_2) becomes indefinitely large, the required N_1 value can at most be reduced only by a factor of 2. Furthermore, this reduction in required diseased cases may be inappropriate if one wishes to obtain clear results for the separate subcategories.

The study size requirements suggested by the information formula may be seriously in error if the binomial parameters show excessive variation among subcategories. Ordinary precautions, however, should serve to keep the formula useful. In some situations it may be desirable to modify the information formula indicated above to reflect the contribution due to variation in the binomial parameters involved.

The second statistical procedure involves setting reasonable limits on the relative risk when it is in fact different from unity. For the homogeneous case considered, formulas for such limits have been published in (*46*). The chi-square test as stated is essentially a test of whether or not the confidence limits include unity. Extension of this procedure to more complex cases is fairly involved and depends primarily on the measure of relative risk adopted. In the absence of a clear justification for any single measure of over-all relative risk, the burden of extremely involved computation of confidence limits in such cases would not seem warranted. Instead, we feel that emphasis should be directed to obtaining an over-all measure of risk, coupled with an over-all test of statistical significance.

Statistical Procedures for Factor Control

A major problem in any epidemiological study is the avoidance of spurious associations. It has been remarked that where the risk of disease changes with age, apparent association of the disease with other age-related factors can result. However, there are appropriate statistical procedures for controlling those factors known or suspected to be related to disease occurrence. They serve not only to remove bias from the investigation but, in addition, can add to its precision.

Two simple procedures for obtaining factor control may first be mentioned. One is simply to restrict the investigation to individuals homogeneous on the factors to be controlled. For this situation the statistical procedures already outlined would be appropriate. The potential number of individuals available for such a study would, of course, be sharply restricted.

There is also the matching case method. A sample of N diseased individuals is drawn and the characteristics of each individual noted with respect to the control factors. Subsequently, a sample of N well individuals is drawn, with each individual matched on the control factors to one of the diseased individuals. The statistical procedures to be presented can be shown to cover the matched-sample approach as a special case, and a discussion of the analysis of such data will be given in that context. Some difficulties of the matched-sample study may be mentioned here. One is that when matching is made on a large number of factors, not even the fiction of a random sampling of control individuals can be maintained. Instead, one must be grateful for each matching control available. Another difficulty is that the method cannot be applied to factors under control, since diseased and control individuals are identical with respect to these factors. Conversely, factors under study in matched samples cannot themselves be controlled statistically. They can be analyzed separately or in particular conjunctions but cannot be employed as control factors.

An alternative to case matching is to draw independent samples of cases and controls, and adjust for other factors in the analysis. This approach requires simply the classification of individuals according to the various control and study factors desired, and an analysis for each separate subclassification as well as an appropriate summary analysis. Its success will depend on a reasonable degree of cross-matching between observations on diseased and control persons. In a small study various devices for reducing the number of subclassifications and for increasing the chances of cross-matching may be necessary, including a limit on the number of factors on which individuals are classified in any one analysis and the use of broad categories for any particular classification. Thus, a 10-year interval for age classification might permit a reasonable degree of cross-matching, whereas a 1-month interval would not.

The need for some degree of deliberate matching, even when the classification approach is employed, can be seen. If the disease under consideration occurs at advanced ages, little cross-matching would result

if controls were selected from the general population. The remedy lies in deliberately selecting controls from the same age groups anticipated for persons with the disease, perhaps even matching one or more controls on age for each diseased person. This principle can be extended to matching on several control factors, *solely for the purpose of increasing the extent of cross-matching in the analysis.*

One of the subtle effects which can occur in a retrospective study, even with careful planning, may be pointed out. It can be shown, for instance, that within a given age interval the average age of individuals with cancer of certain sites will be greater than the average age of individuals from the general population in the same age interval. This can arise when incidence increases rapidly with age and may pose a serious problem with broad age intervals. This effect can be offset by close matching of cases and controls on age in drawing samples, even though they are classified by a broad age category in the analysis.

When a random sample of diseased and disease-free individuals is classified according to various control factors the distribution of the factor under study within the *ith* classification may be represented as follows:

	With factor	Free of factor	Total
With disease	A_i	B_i	N_{1i}
Free of disease	C_i	D_i	N_{2i}
Total	M_{1i}	M_{2i}	T_i

Within this subgroup the approximate relative risk associated with the disease may be written as A_iD_i/B_iC_i. One may compare the observed number of diseased persons having the factor, A_i, with its expectation under the hypothesis of a relative risk of unity, $E(A_i)=N_{1i}M_{1i}/T_i$. The discrepancy between A_i and $E(A_i)$ (which is also the discrepancy for any other cell within a 2×2 table) can be tested relative to its variance which, subject to the fixed marginal totals—N_{1i}, N_{2i}, M_{1i}, and M_{2i}—is given by $V(A_i) = N_{1i}N_{2i}M_{1i}M_{2i}/T_i^2(T_i-1)$. The corrected chi square with 1 degree of freedom $(|A_i-E(A_i)|-\frac{1}{2})^2/V(A_i)$ reduces in this case to $(|A_iD_i-B_iC_i|-\frac{1}{2}T_i)^2(T_i-1)/N_{1i}N_{2i}M_{1i}M_{2i}$. This formula for the variance of A_i is obtained as the variance of the binomial variable $N_1PQ(P = M_1/T, Q = M_2/T)$, multiplied by a finite population correction factor $(T-N_1)/(T-1) = N_2/(T-1)$. The earlier chi-square formula, which is ordinarily used, essentially employs a finite population correction factor of N_2/T.

There is thus a difference between the two chi-square formulas of a factor of $(T-1)/T$ which, though trivial for any single significance test with respectably large T, can become important in the over-all significance test. It is with the latter formula, just presented, that chi square is computed as the ratio of the square of a deviation from its expected value to its variance.

The adjustment for control factors is at this point resolved for the resulting separate subclassifications. The problem of over-all measures of relative risk and statistical significance still remains. A reasonable over-all

significance test which has power for alternative hypotheses, where there is a consistent association in the same direction over the various subclassifications between the disease and a study factor, is provided by relating the summation of the discrepancy between observation and expectation to its variance. The corrected chi square with 1 degree of freedom then becomes $(|\Sigma A_i - \Sigma E(A_i)| - \frac{1}{2})^2/\Sigma V(A_i)$ where $E(A_i)$ and $V(A_i)$ are defined as above.

The specification of a summary estimate of the relative risk associated with a factor is not so readily resolved as that for an over-all significance test, and involves consideration of alternate approaches to a weighted average of the approximate relative risks for each subclassification (A_iD_i/B_iC_i). If one could assume that the increased relative risk associated with a factor was constant over all subclassifications, the estimation problem would reduce to weighting the several subclassification estimates according to their respective precisions. The complex maximum likelihood iterative procedure necessary for obtaining such a weighted estimate would seem to be unjustified, since the assumption of a constant relative risk can be discarded as usually untenable.

Another possible criterion for obtaining a summary estimate of relative risk would involve weighting the risks for subclassification by "importance." A twofold increase of a large risk is more important than a twofold increase of a small risk. An increased risk for a large group is more important than one for a small group. An increased risk for young individuals may be more important than for older individuals with a shorter life expectation. Difficulties arise in attempts to weight relative risk by measures of importance. For one, the necessary information on importance, in terms of the size of the populations affected or in terms of the absolute level of rates prevailing in the subgroups, is generally not contained within the scope of the investigation. A problem in definition of the precise terms of the weighted comparison also appears. Does one want to adjust the risks of disease among persons with the factor to the distribution of the population without the factor, or *vice versa*, or adjust the risks for the populations with and without the factor to a combined standard population? These procedures, and the different phrasing of the comparisons which they entail, could yield different answers. If only a small proportion of the population with the factor was in a subcategory with a high relative risk, while most of the factor-free population fell into this subcategory, and in other categories the relative risk associated with the factor was less than unity, the factor would appear to exert a protective influence under one set of weights but a harmful effect under the other.

Published instances of summary relative risks do not fall clearly into either of the two categories—weighting by precision or weighting by importance. They do follow an approach usually employed in age-adjusting mortality data. Since the relative risk for a single 2×2 table can be obtained from the incidence of the factor among diseased and well individuals, the problem would appear translatable into terms of obtaining

over-all, category-adjusted incidence figures. Direct or indirect methods of adjustment can be used, employing as a standard of reference the frequency distribution or rates corresponding to the sample of diseased persons, of controls, or the diseased persons and controls combined.

While such adjustment procedures provide weighting by importance in their customary application to mortality rates, this is not so in the relative risk situation. This may be illustrated in the following extreme example. Suppose that in each of two subcategories the approximate relative risk for a contrast between the presence and absence of a factor is about 5, which arises in the first subcategory from contrasting percentages of 1 and 5, and in the second subcategory from contrasting percentages of 95 and 99. If these percentages were based on equal numbers of individuals, all methods of category adjusting would yield contrasting adjusted summary percentages of 46 and 52, and a resultant relative risk of slightly less than 1.3. Some other approach for obtaining category-adjusted relative risks would seem desirable. However, to the extent that such extreme situations are not encountered in actual practice, results based on these more conventional adjustment procedures will not be grossly in error.

A suggested compromise formula for over-all relative risk is given by $R = \Sigma(A_iD_i/T_i)/\Sigma(B_iC_i/T_i)$. As a weighted average of relative risks this formula would, in the illustration given, yield the over-all relative risk of 5 found in each of the two subcategories. The weights are of the order $N_{1i}N_{2i}/(N_{1i} + N_{2i})$ and as such can be considered to weight approximately according to the precision of the relative risks for each subcategory. The weights can also be regarded as providing a reasonable weighting by importance.

An interesting property of this summary relative risk formula is that it equals unity only when $\Sigma A_i = \Sigma E(A_i)$ and hence the corresponding chi square is zero. From the fact that $A_i - E(A_i) = (A_iD_i - B_iC_i)/T_i$, it follows that when $\Sigma A_i = \Sigma E(A_i)$, $\Sigma A_iD_i/T_i$ will equal $\Sigma B_iC_i/T_i$, chi square will be zero, and R will be unity. The chi-square significance test can thus be construed as a significance test of the departure of R from unity.

Of some other procedures for measuring over-all relative risks, the one following also has the interesting property of being equal to unity when $\Sigma(A_i) = \Sigma E(A_i)$ and therefore subject to the chi-square test:

$$R_1 = \frac{\Sigma A_i \Sigma D_i}{\Sigma B_i \Sigma C_i} \Big/ \frac{\Sigma E(A_i) \Sigma E(D_i)}{\Sigma E(B_i) \Sigma E(C_i)} \text{ where } E(A_i) = N_{1i}M_{1i}/T_i,\ E(B_i)$$

$$= N_{1i}M_{2i}/T_i,\ E(C_i) = N_{2i}M_{1i}/T_i, \text{ and } E(D_i) = N_{2i}M_{2i}/T_i.$$

In this formula the numerator represents the crude value for the relative risk, which would result from pooling the data into one table and ignoring all subclassification on other factors. The denominator represents the crude value for relative risk, which would have resulted from pooling in the situation where all relative risks within each subclassification were exactly unity. Readers familiar with the "indirect" method of com-

puting standardized mortality ratios will recognize an analogy between the "indirect" method and the above procedure.

The estimator R_1 can be seen to have a bias toward unity. One reason is covered by the illustration which indicated that adjusted percentages (or frequencies) do not yield an appropriate adjusted relative risk. In addition, when either cases or controls have little representation in a subcategory, there will be lack of cross-matching and little information about relative risk, and the observed cell frequencies and their expectations will be numerically close. Such results will, in the process of summation used by the estimator, tend to force its value toward unity. This weakness will not be too important if the degree of cross-matching is roughly equal in the various subclassifications—an optimum goal one would normally attempt to achieve. The bias will become more pronounced as the number of control factors increases and as the prospects for good cross-matching become poorer.

We used the estimator R_1 in a recent paper (*27*), knowing its potential weaknesses. This was done to present results more nearly comparable with those reported by other investigators using similarly biased estimators. One set of results from this paper on lung cancer among women illustrates the conservative behavior of estimator R_1 compared with R, as additional factors are controlled. The relative risk (R_1) for epidermoid and undifferentiated pulmonary carcinoma associated with smoking more than one pack of cigarettes daily as compared to nonsmokers decreased from 7.1 (controlled for age) to 5.6 (controlled for age and coffee consumption). The corresponding figures, with R as a measure of relative risk, were 9.7 and 9.9.

Computational procedures for R and R_1 are presented in table 1, drawing on material comparing smoking histories of women diagnosed as cases of epidermoid and undifferentiated pulmonary carcinoma with those of female controls. For simplicity in presentation only two smoking levels are considered—nonsmokers and smokers of more than one pack of cigarettes daily. An extension of the significance testing procedures to the case of study factors at more than two levels is discussed later. The control factors are age and occupation. The basic data are given in the first 9 columns. Columns 10 and 11 carry the derivative calculations required for R. Columns 12 and 13 are used in the computation for R_1 and for the variance estimate in column 14—the latter being needed for the chi-square test. Only columns 1 to 10, 12, and 14 would be necessary to compute chi square, R and R_1. Column 13 is not essential for the computation of $E(D)$ but simplifies computation of $V(A)$, while providing a check on $E(A)$. Column 11 serves as a check on 10 and 12. A system of checks and computations is outlined at the bottom of table 1. Not all the computations shown would ordinarily be necessary for an analysis.

The corrected chi-square value of 30.66 (1 degree of freedom) would indicate a highly significant association between epidermoid and undifferentiated pulmonary carcinoma and cigarette smoking in women, after adjusting for possible effects connected with age or occupation. The

TABLE 1.—*Illustrative computations for chi square and for summary measures of undifferentiated pulmonary carcinoma*

Group		Epidermoid-undifferentiated pulmonary carcinoma			Controls			Cases and controls		
		1 + Pack cigarettes daily	Nonsmokers	Total	1 + Pack cigarettes daily	Nonsmokers	Total	1 + Pack cigarettes daily	Nonsmokers	Total
		A	B	N_1	C	D	N_2	M_1	M_2	T
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Housewives	under age 45	0	2	2	0	7	7	0	9	9
	45–54	2	5	7	1	24	25	3	29	32
	55–64	3	6	9	0	49	49	3	55	58
	65 and over	0	11	11	0	42	42	0	53	53
White-collar workers	under age 45	3	0	3	2	6	8	5	6	11
	45–54	2	2	4	2	18	20	4	20	24
	55–64	2	4	6	2	23	25	4	27	31
	65 and over	0	6	6	1	11	12	1	17	18
Other occupations	under age 45	1	0	1	3	10	13	4	10	14
	45–54	4	1	5	1	12	13	5	13	18
	55–64	0	6	6	1	19	20	1	25	26
	65 and over	1	3	4	0	15	15	1	18	19
Total		18	46	64	13	236	249	31	282	313

Checks: Total discrepancy, $Y, = \Sigma A - \Sigma E(A) = \Sigma(1) - \Sigma(12) = 11.625$
$= \Sigma D - \Sigma E(D) = \Sigma(5) - \Sigma(13) = 11.625$
$= \Sigma(AD/T) - \Sigma(BC/T) = \Sigma(10) - \Sigma(11) = 11.625$

$\Sigma(15) + \Sigma(16) = 64.000$; $\Sigma(3) = 64$
$\Sigma(17) + \Sigma(18) = 249.000$; $\Sigma(6) = 249$

Derivative computations: $\Sigma E(B) = \Sigma(2) + Y = 57.625$
$\Sigma E(C) = \Sigma(4) + Y = 24.625$
$\Sigma(AT/N_1) = \Sigma(1) + \Sigma(17) = 94.960$
$\Sigma(BT/N_1) = \Sigma(2) + \Sigma(18) = 218.040$
$\Sigma(CT/N_2) = \Sigma(4) + \Sigma(15) = 16.325$
$\Sigma(DT/N_2) = \Sigma(5) + \Sigma(16) = 296.675$

value of R implies that the risk of these cancers is 10.7 times as great for women currently smoking in excess of 1 pack a day than for women who never used cigarettes. The value of R_1, 7.05, is almost identical with the crude relative risk, 7.10, which results from pooling the data with no attention to the control factors. The difference from the published R_1 value of 6.3 in (*27*) arises from the exclusion in the illustrative example, of data for women currently smoking 1 pack a day or less and for occasional or discontinued smokers.

The computation of three other summary estimates of relative risk is also outlined in table 1. The additional derivative computations required for this purpose appear in columns 15 to 18. All three estimates are based on a direct method of category adjustment, that is, the use of a standard distribution to which both the case and control distributions are

relative risk (R, R_1, R_2, R_3, *and* R_4) *relating to the association of epidermoid and in women with smoking history*

Derivative computations								
$\frac{AD}{T}$ $\frac{(1)(5)}{(9)}$ (10)	$\frac{BC}{T}$ $\frac{(2)(4)}{(9)}$ (11)	$\frac{E(A)}{(3)(7)}$ $\frac{}{(9)}$ (12)	$\frac{E(D)}{(6)(8)}$ $\frac{}{(9)}$ (13)	$\frac{V(A)}{(12)(13)}$ $\frac{}{(9)-1.0}$ (14)	$\frac{N_1C}{N_2}$ $\frac{(3)(4)}{(6)}$ (15)	$\frac{N_1D}{N_2}$ $\frac{(3)(5)}{(6)}$ (16)	$\frac{N_2A}{N_1}$ $\frac{(1)(6)}{(3)}$ (17)	$\frac{N_2B}{N_1}$ $\frac{(2)(6)}{(3)}$ (18)
0	0	0	7. 000	0	0	2. 000	0	7. 000
1. 500	0. 156	0. 656	22. 656	0. 480	0. 280	6. 720	7. 143	17. 857
2. 534	0	0. 466	46. 466	0. 380	0	9. 000	16. 333	32. 667
0	0	0	42. 000	0	0	11. 000	0	42. 000
1. 636	0	1. 364	4. 364	0. 595	0. 750	2. 250	8. 000	0
1. 500	0. 167	0. 667	16. 667	0. 483	0. 400	3. 600	10. 000	10. 000
1. 484	0. 258	0. 774	21. 774	0. 562	0. 480	5. 520	8. 333	16. 667
0	0. 333	0. 333	11. 333	0. 222	0. 500	5. 500	0	12. 000
0. 714	0	0. 286	9. 286	0. 204	0. 231	. 769	13. 000	0
2. 667	0. 056	1. 389	9. 389	0. 767	0. 385	4. 615	10. 400	2. 600
0	0. 231	0. 231	19. 231	0. 178	0. 300	5. 700	0	20. 000
0. 790	0	0. 211	14. 211	0. 166	0	4. 000	3. 750	11. 250
12. 825	1. 201	6. 375	224. 375	4. 036	3. 325	60. 675	76. 960	172. 040

Chi-square: $X^2 = (|\text{discrepancy}| - 0.5)^2/\Sigma V(A) = (|Y| - 0.5)^2/\Sigma(14) = 30.66$

Relative risk: $R = \Sigma(AD/T)/\Sigma(BC/T) = \Sigma(10)/\Sigma(11) = 10.68$

R_1 { crude relative risk, $r = \Sigma A\Sigma D/\Sigma B\Sigma C = \Sigma(1)\Sigma(5)/\Sigma(2)\Sigma(4) = 7.10$
adjustment factor, $f = \Sigma E(A)\Sigma E(D)/\Sigma E(B)\Sigma E(C) = \Sigma(12\Sigma(13)/\Sigma E(B)\Sigma E(C) = 1.0081$
$R_1 = r/f = 7.05$

$R_2 = \Sigma A\Sigma(N_1D/N_2)/\Sigma B\Sigma(N_1C/N_2) = \Sigma(1)\Sigma(16)/\Sigma(2)\Sigma(15) = 7.14$

$R_3 = \Sigma(N_2A/N_1)\Sigma D/\Sigma(N_2B/N_1)\Sigma C = \Sigma(5)\Sigma(17)/\Sigma(4)\Sigma(18) = 8.12$

$R_4 = \Sigma(AT/N_1)\Sigma(DT/N_2)/\Sigma(BT/N_1)\Sigma(CT/N_2) = 7.91$

Note: Figures shown are rounded from those actually calculated and consequently are not fully consistent. Column totals and figures shown do not necessarily agree.

adjusted. If the distribution of diseased cases is taken as the standard distribution to which the controls are adjusted, the estimator becomes

$$R_2 = \frac{\Sigma A_i \Sigma \left(D_i \times \frac{N_{1i}}{N_{2i}} \right)}{\Sigma B_i \Sigma \left(C_i \times \frac{N_{1i}}{N_{2i}} \right)}.$$

Estimator R_2 was used by Wynder *et al.* in a study of the association of cervical cancer in women with circumcision status of sex partners (*16*). The merit of employing the cervical cancer case-distribution as the standard presumably rests on the fact that this distribution at least would be well defined by the study.

If the distribution of control cases is taken as standard the estimator becomes

$$R_3 = \frac{\Sigma\left(A_i \times \frac{N_{2i}}{N_{1i}}\right)\Sigma D_i}{\Sigma\left(B_i \times \frac{N_{2i}}{N_{1i}}\right)\Sigma C_i}.$$

If the combined distribution is taken as standard the estimator becomes

$$R_4 = \frac{\Sigma\left(A_i \times \frac{T_i}{N_{1i}}\right)\Sigma\left(D_i \times \frac{T_i}{N_{2i}}\right)}{\Sigma\left(B_i \times \frac{T_i}{N_{1i}}\right)\Sigma\left(C_i \times \frac{T_i}{N_{2i}}\right)}.$$

If any N_{1i} or N_{2i} should equal zero, the estimator R_4 would not be defined. R_2 is not defined for any zero-valued N_{2i}, and R_3 is not defined for any zero-valued N_{1i}. In these instances it would be necessary to exclude the zero-frequency categories to define the estimators. The estimator R_1 retains these categories at the expense of greater bias toward unity. The estimator R gives such categories zero weight, since they contain no information about relative risk. The chi-square significance test gives no weight to these categories.

While R_4 is clearly a direct adjusted estimate of relative risk employing the combined distribution as standard, R_2 and R_3 may be viewed alternatively as either direct or indirect adjusted estimates. The same estimates will result if a direct adjustment is made using the distribution of cases as standard, or an indirect adjustment is made using the factor incidence rates for controls as the standard rates.

It may be noted that in the example used, the values for R_2, R_3, and R_4 (7.14, 8.12, and 7.91, respectively) were roughly comparable to R_1, and all were smaller than R. The example was selected because all the N_{1i} and N_{2i} values were non-zero, so that the values of R_2, R_3, and R_4 were all defined.

The over-all relative risk estimates are averages and as averages may conceal substantial variation in the magnitudes of the relative risk among subgroups. Ordinarily, the individual subcategory data should be examined, paying special attention to relative risks based on reasonably large sample sizes. This will provide protection against the potential deficiencies of any particular summary relative risk formula employed. The over-all chi-square significance test in any case will remain appropriate for detecting any strong general tendency for the risk of disease to be associated with the presence or absence of the test factor.

The Matched-Sample Study

The matched-sample study previously described can be considered a special case of the classification procedure with the number of classifications equal to the number of pairs of individuals. The status of pairs of well and diseased individuals classified with respect to the presence or absence of the suspect factor in each individual will be represented as

F, G, H, or J in the following fourfold table. The meanings attached to the marginal totals A, B, C, and D are the same as those in the first schematic representation.

	Diseased individuals		
Well individuals	With factor	Free of factor	Total
With factor	F	G	C
Free of factor	H	J	D
Total	A	B	N

In the absence of association between the disease and the factor, we expect the same number of individuals with the factor to appear among both diseased and well individuals; that is, we expect $A(=F+H)$ to equal $C(=F+G)$. This can occur only when $G=H$ and the statistical test is simply whether or not G differs significantly from 50 percent of $G+H$. G is tested as a binomial variable with parameter ½, $G+H$ being the number of cases. G thus has expectation ½$(G+H)$, variance ¼$(G+H)$ and the corrected chi square with 1 degree of freedom can readily be shown to reduce to $(|G-H|-1)^2/(G+H)$.

Treating the data as consisting of N classifications each with $N_{1i}=N_{2i}=1$, $T_i=2$ and applying the previously described procedures will lead to the same value of chi square. For F of the N classifications, $A_i=1, M_{1i}=2, M_{2i}=0, E(A_i)=1, V(A_i)=0$; for G classifications $A_i=0, M_{1i}=M_{2i}=1, E(A_i)=½, V(A_i)=¼$; for H classifications $A_i=1, M_{1i}=M_{2i}=1, E(A_i)=½, V(A_i)=¼$; and for J classifications, $A_i=0, M_{1i}=0, M_{2i}=2, E(A_i)=0, V(A_i)=0$. Thus, $\Sigma A_i=F+H$, $\Sigma E(A_i)=F+½(G+H)$, $\Sigma V(A_i)=¼(G+H)$, and the resultant corrected chi square can again be seen to be $(|G-H|-1)^2/(G+H)$.

It is of interest to observe that the summary chi-square formula is appropriate in the matched-sample case, even though the frequencies for each of the separate subclassifications are small. Its appropriateness, despite the small frequencies, stems from the fact that it is a test on a summation of random variables, A_i, and thus tends to approach normality rapidly, making the chi-square test valid, even though the individual A_i's are not normally distributed. This property of the chi-square formula applies in the general classification as well as the matched-sample situation. Only substantial lack of cross-matching in the general case would tend to make the chi-square test invalid. It is also essential, of course, that there be some appreciable variation in the presence or absence of the factor under study.

It should be noted that in the matched-sample study with $T_i=2$ for each of the N pairs of individuals, the variances of the A_i's would have been understated by a factor of 2, had $T-1$ been replaced by T in the variance formulas. The usual formula for chi square does essentially make this replacement, but it is usually of little consequence if T is of any reasonable magnitude. The formulas for relative risk in the matched-sample study reduce simply to the following: $R=H/G$; $R_1=R_2=R_3=R_4=AD/BC$.

Study Factors at More Than Two Levels

The preceding discussion on the analysis of retrospective data has been in terms of the test factor under study taking only two values. This framework has sufficed for discussion of the underlying statistical ideas and issues. In practice, the study factor will frequently take on more than two, perhaps many, potential values. When the number of study factor values is large, grouping can reduce them to manageable proportions.

The need to consider only a limited number of classes for the study factor stems from the fact that, when an association is anticipated, most of the significant information about the association will come from the results for the more extreme values of the study factor. While it is efficient to concentrate attention on the test factor classes expected to show the greatest differences in association with the disease, it is also profitable to consider intermediate values for the test factor to seek evidence for a consistent pattern of association. For example, in table 1, a highly significant difference between nonsmokers and women currently smoking more than 1 pack of cigarettes daily was illustrated. Inclusion of data for smokers of 1 pack or less a day showing results intermediate between the other classes would have added little, if anything, to the statistical significance of the results, and might actually lower it, if one made an over-all test of the differences among the three smoking classes. However, the observation that the intermediate smoking class does, in fact, show an intermediate relative risk contributes to an orderly pattern and increases our confidence in the conclusions suggested by the data for the remaining two classes.

For any two particular test-factor levels, the relative risk for one over the other may be calculated using only the data pertaining to those two levels or by using the results for all test levels. In the formulas previously given for R, R_1, R_2, R_3, and R_4, the difference between the two calculating procedures is simply one of setting the values of N_{1i}, N_{2i}, and $T_i = N_{1i} + N_{2i}$ in terms of number of cases and controls occurring at the two study-factor levels only, or defining them in terms of total number of cases and controls in the entire study. When total cases and controls are used in defining N_{1i}, N_{2i}, and T_i, it can be shown that for R_1, R_2, R_3, and R_4 the various relative risks will be internally consistent with each other. If the relative risk for the first level is twice that for the second level, which in turn is twice that for the third level, then the relative risk for the first level will be four times that of the third. These exact relationships do not hold for R as an estimator of relative risk, and a somewhat sophisticated extension of the formula for R would be required to secure this property.

The problem of obtaining a summary chi square when the study factor is at more than two levels is complicated by the fact that the deviations from expectation at the various study-factor levels are intercorrelated. When there are but two levels, the two deviations will have perfect negative correlation, and attention need be directed to only one of the devia-

tions. Irrespective of the number of levels, at any one level the deviation from expectation among diseased persons will be equal, but opposite in sign, to the deviation from expectation among controls, so that attention can be confined to the deviations for diseased persons.

The problem can be stated as one of reducing a set of correlated deviations into a summary chi square. Table 2 applies this process for obtaining a summary chi square to the study of the association of epidermoid and undifferentiated pulmonary carcinoma in women and maximum cigarette-smoking rate, classified into three levels, after adjustment for age and occupation.

The general expressions for the expectations and variances of the number of cases at a particular test-factor level are given in the lower right section of table 2. Also shown is the expression for the covariance between the number of cases at two different test-factor levels. Since the total of all the deviations is zero, one would in general need the variances of, and covariances between, the number of cases at all but one of the levels. The number of covariance terms will rise sharply as the number of test levels are increased. At 3 test levels, there are 2 variance terms and 1 covariance term, while at 10 test levels, there would be 9 variances and 36 covariance terms of interest.

For the general case the burden of computation could be heavy. After all the necessary computation for the deviations, their variances and covariances, there would still remain the problem of converting these, presumably by matrix methods, into a summary chi square. Since the retrospective problem will normally involve only a limited number of test-factor levels, precise procedures will be given only for the three-level situation, and approximate procedures outlined for the general case.

The exact computation procedure for the three-level case is detailed in table 2. Lines (1), (2), and (4) show the total observed and expected frequencies and variances of the number of cases (and controls) at each of the three smoking-rate levels, after adjusting for age and occupation. These are the summary totals over each subclassification obtained by application of the formulas appearing in table 2.

Lines (5) and (6) give the chi squares corresponding to the total deviation from expectation at each of the smoking-rate levels. The chi squares in line (5) are corrected for continuity. They relate to the difference of the particular level to which they apply, from the two other levels combined. Following the usual practice of making no continuity corrections when chi squares with more than 1 degree of freedom are under consideration, line (6) shows the uncorrected chi squares.

The computing procedure of table 2 takes advantage of the fact that, since the sum of the deviations from expectation is zero, the variance of the third deviation must equal the sum of the other two variances plus twice the covariance for the first two deviations. The covariance of the first two deviations is readily obtained as illustrated and is used in calculating the summary chi square. The summary chi square is obtained as the sum of squares of two orthogonal deviates, with each

TABLE 2.—*Illustrative computation of summary chi square, when there are 3 levels for study factor. The data relate to the association of epidermoid and undifferentiated pulmonary carcinoma in women with smoking history*

	1+ Pack cigarettes daily			1 Pack or less of cigarettes daily			Occasional or nonsmokers			Total		
	Epidermoid-undifferentiated pulmonary carcinoma	Controls	Total (ΣM_1)	Epidermoid-undifferentiated pulmonary carcinoma	Controls	Total (ΣM_2)	Epidermoid-undifferentiated pulmonary carcinoma	Controls	Total (ΣM_3)	Epidermoid-undifferentiated pulmonary carcinoma (ΣN_1)	Controls (ΣN_2)	Total (ΣT)
(1) Total observed frequencies	19	17	36	32	71	103	51	251	302	102	339	441
(2) Total expected frequencies, adjusted for age and occupation	9.09	26.91	36	23.76	79.24	103	69.15	232.85	302	102	339	441
(3) Total deviation from expectation (1)−(2)	$+9.91 = Y_1$			$+8.24 = Y_2$			$-18.15 = Y_3$					
(4) Variance of total observed frequencies, subject to fixed marginal totals in each age and occupation group	$5.9163 = V_1$			$12.2900 = V_2$			$14.0723 = V_3$					
(5) Individual corrected chi squares $(\lvert Y\rvert-0.5)^2/V$	$14.97 = X^2_{1c}$			$4.88 = X^2_{2c}$			$22.15 = X^2_{3c}$					
(6) Individual uncorrected chi squares Y^2/V	$16.60 = X^2_1$			$5.53 = X^2_2$			$23.42 = X^2_3$					
(7) Covariance (Y_1, Y_2) $(V_3-V_1-V_2)/2$				-2.0670								
(8) Adjusted Y_2 $Y_2-(7)Y_1/V_1$				11.70								
(9) Adjusted V_2 $V_2-(7)^2/V_1$				11.5678								
(10) Adjusted X^2_2 $(8)^2/(9)$				$11.83 = X^2_2$(ad.)								
(11) Summary chi square (2 degrees of freedom) $X^2_1 + X^2_2$(ad.)				$16.60 + 11.83 = 28.43$								

For the general situation the total expected case frequency at the *jth* level of a test factor is

$$\sum_i N_{1i}M_{ji}/T_i$$

The variance of the total case frequency is

$$V_j=\sum_i \frac{N_{1i}N_{2i}M_{ji}(T_i-M_{ji})}{T_i^2(T_i-1)}$$

The covariance of the total case frequencies at test levels j and k is

$$-\sum_i \frac{N_{1i}N_{2i}M_{ji}M_{ki}}{T_i^2(T_i-1)}$$

The index of summation, i, represents the various subclassifications into which the results are divided

For 3 test levels only, since $Y_3=-(Y_1+Y_2)$, it follows that $V_3=V_1+V_2+2$ Covariance (Y_1, Y_2)

square adjusted for its own variance. The first deviate squared is simply the uncorrected chi square at the first level in line (6)—the variance of the deviate remaining as initially calculated. The second deviate is the deviation at the second level adjusted for its correlation with the first deviation [adjusted $Y_2 = Y_2 - b_{21}Y_1$; b_{21} = covariance (Y_1,Y_2)/variance Y_1)]. The variance of the adjusted second deviate is the initial value reduced by that portion of the variation accounted for by the first deviation [Var. (adjusted Y_2) = variance Y_2 − covariance$^2(Y_1,Y_2)$/variance Y_1)].

In the present instance the summary chi square with 2 degrees of freedom is 28.43 [line (11)]. This presumably is close to the chi square with 1 degree of freedom which would have obtained had only the two most extreme smoking classes been compared. If one examines the individual uncorrected chi squares [line (6)], their total is found to be 45.55, the maximum individual figure being 23.42. *It will necessarily be true that the summary chi-square value will lie between the largest of the three chi squares and their total. At almost any reasonable probability level these limits would be sufficient to establish statistical significance without further calculation.* In our companion paper (*27*) this rule sufficed in almost all instances to separate the significant from the nonsignificant results.

Comments on Extensions to More Than Three Factors

Two procedures can be suggested for getting approximate summary chi squares, when there are a large number of levels for the test factors, without the burden of computation that the exact method would entail. Both methods calculate the approximate summary chi square as a sum of squares of approximately orthogonal standardized deviates.

In the first method one computes an uncorrected chi square with 1 degree of freedom for the difference of the first level from all the remaining levels combined (the same first step as in the illustration for the three-level case). Discarding the data from the first level, a second chi square is computed for the difference between the second test-factor level and the remaining levels combined. This is done successively up to and including the last two remaining levels. The approximate summary chi square is then the sum of the separate chi squares with the number of degrees of freedom being one less than the number of test levels.

Exactly orthogonal standardized deviates would be obtained if, in the summary analysis, as each successive total deviation from expectation were evaluated, it was adjusted for its multiple regression on the preceding deviations, and then standardized by the adjusted variance. This, of course, would no longer be a simplified approximate procedure. However, it can be shown that for a single classification, in the multiple regression of any deviation from expectation on any subset of deviations, the regression coefficients will all be equal; the multiple regression on the set of deviations will be the same as the simple regression on their sum. The equality of regression coefficients, while holding true exactly for deviations in the separate subclassifications, will hold only approximately for the total

deviations from expectation (it would hold exactly if equal numbers of individuals were observed from level to level at each subclassification). Nevertheless, this result suggests that approximately orthogonal deviates would be obtained if, in evaluating each successive total deviation, it were adjusted for the cumulative total of deviations already evaluated. Computing procedures to accomplish this can readily be devised.

Both approximate chi-square procedures just outlined, which may have merit when more than three groups are being compared simultaneously, should, in theory, yield linear combinations of independent chi squares. While testing the chi-square values obtained as though they were exact is not likely to be too inappropriate, it may be more correct to obtain a modified number of degrees of freedom, along the lines suggested by Satterthwaite (*7*) for problems involving such linear combinations. What the modified number of degrees of freedom would be has not been investigated by us, and it may prove as easy to apply the exact chi-square procedure, indicated later, as to determine the appropriate degrees of freedom for the approximate chi square.

It is of interest that a somewhat similar task of obtaining an appropriate summary chi square appears in the birth-order problems described by Halperin (*48*). There, it was necessary to compare a set of total observations (across family sizes) with a set of total expectations, one for each birth order. Halperin described a matrix-inversion procedure for reducing the set of correlated deviations into a summary chi square. In that problem it can be shown that all the regression coefficients are equal in the multiple regression of the deviation at a particular birth order on the set of deviations at all succeeding birth orders. The second approximate method described previously for the present problem could thus be used exactly for the birth-order problem, permitting simplified computation of chi square. The procedure indicated by Halperin has the advantage of generality and could be applied to the current and related problems, if one obtained all the necessary variances and covariances and inverted the resulting matrix.

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Association of Cervical Cancer With Circumcision of Sexual Partner [1,2]

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Summary

In a study of interviews with both cervical-cancer women patients and controls, we were unable to find an association between having the disease and reporting an uncircumcised husband. Dr. E. L. Wynder had previously reported an association in a study that also used data on circumcision history obtained from interviews with women patients. There may have been differences in asking the questions about circumcision but, if so, it is not clear how the data were affected. An additional, independent, investigation of circumcision as an anatomical fact revealed that Jewish men probably have a maximum degree of anatomically complete circumcision, which occurs in less than half of non-Jewish men who consider themselves circumcised. The unique protection from cervical cancer that Jewish women enjoy is expressed in a risk ratio of a large magnitude, and this protection may be associated with maximum circumcision in their partners. The circumcision study also revealed that one fourth to one third of men naturally may have a partially shortened foreskin, which is similar to that of some men who have been circumcised, but, in an interview study, many of them will be counted as uncircumcised. Consequently an interview study of non-Jewish subjects may fail to identify the fully circumcised and further dilute the results with many "partially circumcised" classified as uncircumcised. A study by direct examination of men seems unavoidable.—**J. Nat. Cancer Inst. 22: 749–764, 1959.**

Since Jewish women have a clear advantage in the infrequency of cervical cancer, the factor or factors involved in their protection may explain a large proportion of the disease in non-Jewish women. The relative incidence of the disease in Jewish women compared to that of non-Jewish women may be on the order of one fifth or less (*2*, *3*). In 1954 Dr. E. L. Wynder and coworkers reported on data from two sources in support of the hypothesis that circumcision of the male is a protective

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[2] A portion of the data in this analysis is from a study supported by research grant CS-9160 from the National Cancer Institute, National Institutes of Health, Public Health Service. The over-all results have been reported by Drs. Edward Jones, Ian Macdonald, and Lester Breslow (*1*).

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factor (*2*). Moslem women in India were shown to have less cervical cancer than Hindu women: The Moslems practice universal circumcision of the male, the Hindus do not. In some American hospitals, interviews with non-Jewish, cervical-cancer patients showed that they were exposed to more uncircumcised husbands than a group of comparable hospital controls was.

While the evidence is consistent, the sources of evidence are associated with different problems. In the Jewish and the Moslem data, there is no question about whether the women actually have circumcised husbands, but the inability to separate other factors, possibly associated with ethnic-group life, remains a problem. In the interview study with non-Jewish women these other correlated factors are presumably not in question, but the data on extent of exposure to uncircumcised partners may have its problems. The circumcision-exposure history was taken largely as reported by the women themselves. We have similar interview data from non-Jewish, cervical-cancer patients and controls in Los Angeles, which do not confirm the results from the Wynder study. Along with a comparison of the two results, this report analyzes some additional independent data on circumcision as an anatomical fact in men. The analysis suggests a study technique required to resolve the discrepancy.

Source of Data on Patients and Controls

The data are from a study not designed solely to test the question of circumcision but to study a large number of variables possibly associated with cervical cancer. During the years 1951–55, 429 patients with cervical cancer were interviewed. The same number of controls were selected by matching on age (within 5 years), race, ever married, and ever parous. A further restriction was made by selecting the patient-control pair from the same hospital (Los Angeles County General Hospital) or the same private or group practice. The interviews, conducted by 3 social workers, covered a broad range of questions including sexual and marital history, social factors, personal habits, and nutrition. The general plan of the study and over-all results are reported in a separate publication (*1*).

The matched controls were selected among patients who sought hospital or medical attention for a variety of ailments and disablements. Table 1 shows their distribution by diagnosis. This selection was made to avoid bias that might be due to a specific disease-associated factor, though we are still left with the familiar problem—the use of patients for population estimates. By contrast, Wynder chose controls largely from other gynecological patients, with the result that other genital-tract malignancies were included with the controls. There is suggestive evidence that some of these malignancies may be associated with characteristics related to the occurrence of cervical cancer, but not necessarily in the same direction.

Status as a county-hospital patient or private-office patient represents roughly 2 socioeconomic levels, and we have adhered to these categories for most of the patient-control comparisons in this analysis.

The original 429 matched pairs of patients and controls were sub-

TABLE 1.—*Diagnosis of patients in the total series of controls* from the Los Angeles County General Hospital and private practice*

Diagnosis	Percent (429 patients)
Malignant neoplasm (skin only)	3
Benign neoplasms	9
Allergic, endocrine, metabolic, etc., diseases	3
Diseases of the nervous system	7
Diseases of the circulatory system	4
Diseases of the digestive system	16
Diseases of the genitourinary system	23
Diseases of the skin	7
Diseases of the bones	4
Fractures and other accidents	19
Other	5

*Before exclusion of Jewish patients, unmarried patients, and patients with carcinoma *in situ*.

sequently reduced in number because it was necessary to make exclusions on certain characteristics. The reasons for exclusions are detailed here. Since controls were not selected to match on religion, the first tabulations disclosed 5 Jewish patients and 21 Jewish controls, and these were excluded. (It is interesting that this distribution is about what one would expect as a result of the relative immunity to the disease by Jewish women.) Thirty-five cases with a diagnosis of carcinoma *in situ* were excluded because of uncertainty as to the subsequent development of the invasive disease. Seven patients and seven controls were excluded from the tables because no sexual history was obtained—usually the patients admitted to no sexual relations. Not shown in the tables, because of inadequate numbers when classified separately on race and medical facility, are 1 patient and 1 control of Indian parentage, 3 Mexican controls in the private-practice group with no Mexican patients for the appropriate contrast, and 1 patient and 1 control who were the only Negro patients in the private-practice group. These 7, Mexican, Indian, and Negro, all had uncircumcised principal sexual partners, and their omission from the tables does not affect the conclusions. The numbers shown in the tables were thereby reduced to 380 patients and 396 controls.[5] Since the exclusions destroyed some of the matched pairs, in order to retain maximum use of the remaining data, it was now necessary to control age, race, and medical-facility source by making new subclassifications of the data. This also provided an opportunity to alter the procedure of controlling for age in order to exercise a more rigorous control on year of birth of the husbands of patients. Originally, the controls had been selected to match the patients on age at the time cervical cancer was first diagnosed. The fact that first diagnosis of cervical cancer was sometimes considerably earlier than the interview made the controls a younger cohort. We therefore introduced an additional code for age as of 1951 in order to have patients and controls in

[5] In the tables that follow, totals may differ by 1 or 2 from those just cited. This happens when subclassification according to relevant variables results in an absence of numbers in 1 of the 2 cross-matched groups.

the same age cohort. We also felt this to be necessary when we found that circumcision was related to year of birth. It was found that the percentage circumcised increased about 1 percent per calendar year among male partners identified by patients and controls (56% for those born after 1910 and 8% for those born before 1870), and this trend will also be evident later in the data of table 7. Subsequently we found that Richard Doll had also reported some data showing the importance of year of birth for circumcision studies (*4*).

Results

The first results reported are for the partner known longest. This partner, for all except about 4 percent of both patients and controls, was a husband. For brevity we will refer to all partners as husbands. In the tables that follow, the observed number of circumcised husbands among patients and controls is compared with the "expected" number.[6]

Table 2 shows no association between cervical cancer and lack of circumcision of the husband. For all cervix cases we found 114 husbands (34.1%) reported circumcised, with 112.38 (33.7%) expected.[7] The results are consistent for each race and medical facility, though for these subgroups the numbers are small.

There were a number of subjects whose circumcision status was unknown, and the distribution for them is shown in table 3. The numbers are large for each group, and somewhat larger for patients than for controls—45 patients and 32 controls with an "unknown" circumcision status. This naturally raises the question of whether the data fail to confirm Wynder's conclusions because the unknown status hides a real association.

One can make various assumptions about how these unknowns influence the results. On the most extreme assumption that all unknown cases had uncircumcised husbands, and all unknown controls had circumcised husbands, the relative risk associated with having an uncircumcised husband is only 1.5 to 1, when the risk associated with circumcised husbands is set at unity. It therefore seems unlikely that the unknown observations have concealed an association, at least of any substantial magnitude.

A separate analysis was made of women married once, and this is shown in table 4. Again we find the number of uncircumcised husbands of

[6] We are indebted to Nathan Mantel, Head of the Experimental Statistics Section, National Cancer Institute, for the methods of testing for statistical significance and determining relative risk. In order to adjust for differences between cancer patients and controls, the "expected" number and the variance were computed for each fourfold table resulting from the simultaneous subclassification of patients and controls on age, race, and medical facility. For a single fourfold table, $\begin{array}{|c|c|}\hline a & b \\ \hline c & d \\ \hline \end{array}\ \begin{matrix} N_1 \\ N_2 \\ M_1 M_2 T \end{matrix}$ in which "a" is the observed number of cervical-cancer patients with circumcised husbands, the formula for the expected number, $E(a)$, is N_1M_1/T and for the variance, $V(a) = N_1N_2M_1M_2/T^2(T-1)$. With summations over all the fourfold tables, the determination of chi square, with 1 degree of freedom, is $(|\Sigma a - \Sigma E(a)| - 0.5)^2/\Sigma V(a)$. The over-all relative risk estimate is determined by $\frac{\Sigma a}{\Sigma c}\frac{\Sigma d}{\Sigma b} + \frac{\Sigma E(a)}{\Sigma E(c)}\frac{\Sigma E(d)}{\Sigma E(b)}$, and is equal to unity when chi square is zero. The rationale for both the significance test and the relative risk determination appears in a paper by Mantel and Haenszel in this issue.

[7] The variance of the discrepancy is 38.22 percent. For all case groups combined, an agreement between observed and expected, as close as that actually obtained, could occur in 10 to 20 percent of samples.

TABLE 2.—*Circumcision status of husband known longest. Patients and controls by medical facility and race*

Patient groups	Number of cervical-cancer patients	Age-standardized expected No. of cancer patients*	Number of controls
County hospital			
White/non-Mexican			
Circumcised	39	38. 74	43
Not circumcised	89	89. 26	97
Mexican			
Circumcised	4	3. 79	4
Not circumcised	21	21. 21	24
Negro			
Circumcised	24	23. 97	27
Not circumcised	37	37. 03	40
Total hospital	214	214. 00	235
Private practice (white)			
Circumcised	47	45. 88	52
Not circumcised	73	74. 12	77
Total private practice	120	120. 00	129
All patients			
Circumcised	114 (34. 1%)	112. 38 (33. 7%)	126
Not circumcised	220 (65. 9%)	221. 62 (66. 3%)	238
Total	334 (100. 0%)	334. 00 (100. 0%)	364

*Expected numbers are computed for each age category. The figures in the table are totals for all age categories.

TABLE 3.—*Circumcision status unknown; patients and controls by race**

Patient groups	Patients		Controls	
	Unknown	Total	Unknown	Total
County hospital				
White/non-Mexican	15 (10. 5)	143 (100)	11 (7. 3)	151 (100)
Mexican	6 (19. 4)	31 (100)	5 (15. 2)	33 (100)
Negro	11 (15. 3)	72 (100)	9 (11. 8)	76 (100)
Private practice				
White	13 (9. 8)	133 (100)	7 (5. 1)	136 (100)
All patients	45 (11. 9)	379 (100)	32 (8. 1)	396 (100)

*Numbers in parentheses = percent.

patients to be closely in agreement with the expected number. The unknown circumcision status is shown in table 5.

Comparison With Wynder's Results

The study by Wynder and associates included data on circumcision status of all husbands in the event of more than 1 marriage, in addition to data on whether the women indicated experience with nonmarital partners, though without circumcision status of the latter. Table 4, which reports on women with 1 marriage, is comparable except that it lacks information on nonmarital partners. On the other hand, table 2 lacks comparability with the Wynder study to the extent of reporting results only for partner known longest when women were married more than once.

TABLE 4.—*Circumcision of husbands among cervical-cancer and control patients married once*

Patient groups	Number of cervical-cancer cases	Age-race-standardized expected numbers of cancer cases	Number of controls
County hospital:			
Circumcised	25	24.16	31
Not circumcised	68	68.84	94
Total	93	93.00	125
Private practice:			
Circumcised	25	24.34	40
Not circumcised	47	47.66	61
Total	72	72.00	101
All patients:			
Circumcised	50 (30.3%)	48.50 (29.4%)	71
Not circumcised	115 (69.7%)	116.50 (70.6%)	155
Total	165 (100%)	165.00 (100%)	226

TABLE 5.—*Circumcision status unknown**

Patient groups	Cases		Controls	
	Unknown	Total	Unknown	Total
County hospital	20 (17.7)	113 (100)	18 (12.5)	143 (100)
Private practice	8 (10.0)	80 (100)	5 (4.7)	106 (100)
All patients	28 (14.5)	193 (100)	23 (9.2)	249 (100)

*Numbers in parentheses = percent.

TABLE 6.—*Circumcision status of first partner*

Circumcision status	Cervical-cancer cases		Expected No. of cancer cases*	Controls	
	Number	Percent		Number	Percent
Circumcised	90	(23.7)	99.51	128	(32.3)
Not circumcised	220	(58.0)	210.49	223	(56.3)
Total	310		310.00	351	
Unknown	69	(18.2)		45	(11.4)
Total	379	(100.0)		396	(100.0)

*Standardized for age, race, and medical facility.

It may be argued that a restriction to husband known longest dilutes the results because the effect of exposure to other uncircumcised partners is ignored. If this has happened, it would seem to mean that the remaining relative risk associated with an uncircumcised partner has thereby been reduced to less than twofold. For, assuming an expected rate of 30 percent circumcision, the sample sizes shown in table 2 are quite large enough to detect, with a high degree of probability (0.95), a difference significant at the 0.05 probability level if the actual relative risk for lack of circumcision is 2 to 1 or greater. On the other hand, the samples are not large enough to detect a difference, with the same assurance, if the

actual risk, considering partner known longest, is as low as 1.5 to 1.[8] This statement on sample size and relative risk applies to the heterogeneous groups taken *in toto* and not to whatever relative risk might prevail by subclasses of age or race.

It may be that exposure to an uncircumcised sexual partner is largely a factor at younger ages, and that the partner known longest is frequently not the partner known when young. Our data on first sexual partner are not completely satisfactory probably because of the early age of the experience, especially for the cervical-cancer patients who are known generally to have first coitus earlier and to remarry more often. Among the cervical-cancer patients, median age at beginning of the relationship with first partner, marital and nonmarital, was 18.7 compared to 20.5 for controls. As table 6 shows, considerably more cervical-cancer patients (18.2%) than controls (11.4%) could not supply information on circumcision status of first partner. And comparison with table 3 reveals that these proportions "unknown" are greater than for longest-term partner.

Table 6 shows that fewer cervical-cancer patients reported circumcised first partners—about 24 percent compared with 32 percent expected. The relative risk associated with having an uncircumcised first partner is 1.3 to 1. However almost equal proportions of uncircumcised partners are reported by both patients and controls, and the complementary difference is made up by the unknowns.

We are not suggesting that 1.3 be taken as an estimate of increased risk because the circumstances of being able to state the circumcision status of first partner may be less favorable for the cervical-cancer patients. We show the result because we are unable to call the issue into question quite as strongly about first partner as about partner known longest. And it may be seen that even if the risk-association does concentrate at an earlier age, the order of magnitude may not be large.

The analysis so far has ignored possible exposure to other uncircumcised partners. To conceive of this exposure as having a bearing on the development of cervical cancer, in the absence of any substantial relative risk shown for longest-term partner, may require a special feature about the hypothesis. The carcinogen, if it is present under the foreskin, *e.g.*, smegma, must be highly potent, for the duration of exposure to these other partners would be less than that for longest-term partner.

The focus of research that would cut through most of the problems involved in studying the multiplicity of partners, including those known

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[8] We are indebted to Nathan Mantel also for providing procedures for testing the adequacy of sample size. If P_1 is the proportion of the general population having a characteristic which is anticipated to produce a C-fold increase in the relative risk of disease, the proportion with the characteristic in the disease population will be $P_2 = CP_1/(1 - P_1 + CP_1)$. The necessary sample size to detect the C-fold increased relative risk was calculated from

$$\sqrt{N} = \frac{T}{P_1 - P_2}\left(\sqrt{2\bar{P}(1-\bar{P})} + \sqrt{P_1(1-P_1) + P_2(1-P_2)}\right) \text{ where } \bar{P} = (P_1+P_2)/2.$$

N is the number of cases and controls needed respectively if one intends testing at a significance level corresponding to the normal deviate T and wishes assurance, at a probability level also corresponding to the normal deviate T, of obtaining a significant finding if the risk of disease has in fact been changed by a factor of C different from unity. In this formulation the difference between the general population and the well population in the occurrence of the characteristic is neglected.

briefly long ago, is the concentration on women who have had only 1 husband. For these women, Wynder found that the risk associated with having an uncircumcised husband relative to the risk for those with circumcised husbands was 2.5 to 1, though his report points out that the sampling error is large (*2*). Our data on 1 marriage, in table 4, fails to show any difference apropos of circumcision between patients and controls. A possible shortcoming in this is that our sample is somewhat meager. For all categories we have 391 patients married once only, while Wynder had 841. However, with these 391 divided into 165 patients and 226 controls, and with an expected circumcision rate of 30 percent, we find the numbers to be sufficient to detect a significant risk, *i.e.*, with 0.95 assurance if the actual relative risk is 2.5 to 1 or greater, but the assurance is not reasonably high if the relative risk is actually as low as 2 to 1. In short, while our sample for single marriages is too small to be sure of finding a low-order risk, our results cast doubt on the idea of a high-order risk, assuming no biases in our data.

Comparison of Methods

The proportion of men reported to be circumcised is greater in our study than in Wynder's. For the total group we found 30.0 percent reported circumcised with 9.9 percent unknown. For the Los Angeles County General Hospital, white males, 27.9 percent were reported circumcised and 8.8 percent unknown. Wynder shows 20.0 percent with circumcised husbands among white women controls married once only, with 8.0 percent unknown, and, in questioning a group of white male patients directly, 16 percent were circumcised (*2*). Did some bias, perhaps in our interviewing technique, overstate the number of circumcised husbands as reported by wives?

In Los Angeles County General Hospital, in 1958, in an independent interview survey of white male patients age 40 and over, which will be given in detail later, 23.9 percent reported they were circumcised; 10.0 percent said they did not know. This compares fairly well with 27.9 and 8.8 percent reported by the women patients, in a study at the same hospital from 3 to 7 years earlier. More Negro women in our study reported circumcised husbands (35.5%), but they were younger than the white patients, and the higher rate may be a consequence of younger husbands.

While the interview procedures may have yielded a slightly higher circumcision rate than if the men themselves had been queried, it may be reasonable to expect a higher rate than Wynder got. Men of foreign birth are less likely to be reported as circumcised. Wynder reports that circumcision is twice as frequent among the native born. Using the women's answers, we found the proportions to be 36.8 percent among those born in the United States and 21.7 percent among foreign born, with the sample containing only 84 foreign born. The Bureau of the Census reports for 1950 show the proportion of foreign born among white males in the Los Angeles Standard Metropolitan Area to be 10.6 percent.

In the New York Standard Metropolitan Area, where 74 percent of Wynder's subjects were hospitalized, the comparable proportion for foreign born was 21.5 percent. (The proportion of foreign-born husbands among patients and controls in the Los Angeles study were 16.3 percent and 14.9 percent, respectively. They were not reported in Wynder's study.) The higher proportion of foreign born in New York than in Los Angeles may represent one possible explanation of the lower circumcision rate among non-Jewish men in New York.

Certain differences in interview technique are clear. Wynder used physicians for some of the interviewing, and at one hospital for which the results are reported separately, the interviewing was "blind." In the present study, interviewers, who were social workers, knew which were patients and which were controls.

Throughout the study, interviewing in Los Angeles was under the direction of a professor of social work, who did the first interviewing herself and then trained another interviewer and the latter's replacement. The interviewing in Los Angeles was almost completed before Wynder's results were known.

Analysis of the Circumcision Status of Men

During 1958, in an endeavor to gain further understanding of the problem, a number of white male patients in Los Angeles County General Hospital were asked about their circumcision status.[9] This was precipitated by our hearing of a study by Lilienfeld and Graham, now published (*5*), in which a marked discrepancy was found between questionnaire response of men as to their circumcision status and the independent findings from examinations by physicians. Our purpose was to check on the validity of interview responses to a question about circumcision. It should be noted that the responses studied were those of men about themselves, not those of women concerning their husbands. There was an opportunity to check on the men's answers by following the interview with a physician's examination. Two physicians did the work, with each examining the other physician's interviewees. The results are for men who were in poor economic circumstances when hospitalized. While this limits generalizations about men, cervical cancer also occurs more frequently among women at this income level.

During the initial trials of the examination, it became clear that a number of patients were difficult to classify. It also became apparent that there were two separate questions: 1) Did the patient, years ago, have the operation he claims? A disagreement between patient and examining physician cannot be settled unilaterally, for the operation of circumcision may not have uniform results. 2) Anatomically, is the patient effectively circumcised? Not only is this question more important

[9] We are indebted to the following physicians who provided the data for this analysis: Dr. Lester Grinspoon, who initiated the work; and Drs. George Drake and Charles Carpenter, who completed the study and whose results are used here. All are, or were, field investigators with the Field Investigations and Demonstrations Branch, of the National Cancer Institute.

for the cervical-cancer hypothesis, it is the only one that can be answered with full confidence by the examination alone.

An intermediate classification of incomplete or partial circumcision was formulated for use in examinations. There were, then, the following criteria for diagnosis of circumcision: 1) Circumcised—defined as a condition in which the foreskin, upon release of forward traction, retracts to a point where no part of the glans penis is covered. 2) Uncircumcised—defined as a condition in which the foreskin at rest or upon release of forward traction completely covers the glans penis or retracts only so far as the tip of the glans penis. 3) Partially circumcised—defined as condition in which foreskin upon release of forward traction covers the glans penis only partially.

Table 7 shows that 24 percent of the non-Jewish men say they are circumcised, while 18 percent show complete circumcision on examination. In table 8, of the men who report they were circumcised, only 48 percent were found on examination to be completely circumcised. Some 7 percent of those reporting "not circumcised" are also found with complete circumcision.

Lilienfeld and Graham, in presenting their data, considered the patients' statements as to circumcision status in relation to what the physician recorded from a physical examination. The more pertinent consideration for the studies we are concerned with here is the anatomical finding in relation to what the individual states his circumcision status to be. Table 9 presents Lilienfeld and Graham's data arranged for comparison with that taken from table 8. Their findings are that one third of those men claiming to be circumcised were judged not to be, by examination; and one third of those who said they were not, were considered to be circumcised, by examination. It is quite obvious, and would be expected by the account of the varying criteria used by the examining physicians in their study, that the group called partially circumcised, in our study, were arbitrarily divided into somewhat equal portions and assigned to

TABLE 7.—*Circumcision status of non-Jewish men at Los Angeles County General Hospital, 1958**

Age group	Determined by patients' reports			Determined by physician's examination		
	Circumcised	Not circumcised	Don't know	Circumcised	Partially circumcised	Not circumcised
All ages, 40+ 184 (100%)	44 (23.9)	122 (66.3)	18 (9.8)	33 (17.9)	69 (37.5)	82 (44.6)
40–49 41 (100%)	17 (41.5)	24 (58.5)	0	9 (22.0)	12 (29.3)	20 (48.8)
50–59 45 (100%)	12 (26.7)	30 (66.7)	3 (6.7)	10 (22.2)	19 (42.2)	16 (35.6)
60–69 49 (100%)	9 (18.4)	33 (67.3)	7 (14.3)	8 (16.3)	17 (34.7)	24 (49.0)
70+ 49 (100%)	6 (12.2)	35 (71.4)	8 (16.3)	6 (12.2)	21 (42.9)	22 (44.9)

*Numbers in parentheses = percent.

circumcised and uncircumcised status, in Lilienfeld and Graham's study. This would be the result, as will be indicated later, if the foreskin coverage of half the glans were used as the point of division for such an assignment of our partially circumcised group. This explanation for reaching comparability serves well for the patients claiming circumcision, though for those claiming not to be circumcised our results would be less (23.8 *vs.* 34.6%) for those considered anatomically circumcised. These percentages would not be significantly different in samples of this size, and the differences in criteria for judging circumcision could lead to some selective differences.

Approximately one third of all non-Jewish men shows partial circumcision, and this proportion is not much greater for those who claim, than for those who do not claim, to be circumcised. Those who claim to be circumcised and show partial retention of the foreskin (39%) provide an interesting point for speculation. Does the operation as performed on non-Jewish males in the United States result in incomplete circumcision in a number of cases, perhaps up to one third? To say that none of this is the incomplete effect of an operation but only incorrect knowledge on

TABLE 8.—*Patients' reports compared with physician's examination for circumcision of non-Jewish white males, Los Angeles County General Hospital, and of Jewish males at the Los Angeles General and Cedars of Lebanon Hospitals, 1958*

Physician's examination	Patients' reports					
	Circumcised		Not circumcised		Don't know	
	Number	Percent	Number	Percent	Number	Percent
Non-Jewish (184 patients)						
Total, age 40+	44	100. 0	122	100. 0	18	100. 0
Circumcised	21	47. 7	8	6. 6	4	22. 2
Partially circumcised	17	38. 6	42	34. 4	10	55. 6
Not circumcised	6	13. 6	72	59. 0	4	22. 2
Total, age 40–59	29	100. 0	54	100. 0	3	
Circumcised	16	55. 2	3	5. 5	0	
Partially circumcised	11	37. 9	17	31. 5	3	
Not circumcised	2	6. 9	34	63. 0	0	
Total, age 60+	15	100. 0	68	100. 0	15	
Circumcised	5	33. 3	5	7. 3	4	
Partially circumcised	6	40. 0	25	36. 8	7	
Not circumcised	4	26. 7	38	55. 9	4	
Jewish (15 patients—L.A.C.G.H.).						
Total, all ages	15					
Circumcised	14					
Partially circumcised	0					
Not circumcised	1					
Jewish (50 patients—C. of L.H.)						
Total, all ages	50					
Circumcised	44					
Partially circumcised	6					
Not circumcised	0					

the part of the men seems unrealistic, since it would follow that 52 percent (38.6 + 13.6) of the men were wrong about their circumcision status. On the other hand, there is some interesting evidence, though based on small numbers, that is somewhat in accord with the speculation that there is a sizable proportion of incomplete circumcisions. Fourteen of the men said they were circumcised at age 4 or later, 5 between age 4 and 9, 5 at age 10 to 19, and 4 between age 20 and 29. One may assume that these men had a better opportunity to recollect that an operation had actually been performed. On examination, 9 were found to be circumcised, 1 uncircumcised, and 4 incompletely circumcised. Variation in the completeness of surgical removal of the foreskin seems certain, and the evidence suggests it is less than complete for a sizable proportion.

By contrast, there seems to be little variation in the removal of foreskin in Jewish men (table 8). Among 15 Jewish men, examined at the Los Angeles County Hospital, circumcision was found in 14, none were par-

TABLE 9.—*Comparison of the anatomical findings in relation to the patients' statement as to his circumcision status, from Lilienfeld and Graham's data and table 8*

Physician's examination	Patients' report							
	Circumcised				Not circumcised			
	Table 8		Lilienfeld		Table 8		Lilienfeld	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total	44	100.0	56	100.0	122	100.0	136	100.0
Circumcised	21	47.7	37	66.1	8	6.6	47	34.6
Partially circumcised	17	38.6	—	—	42	34.4	—	—
Not circumcised	6	13.6	19	33.9	72	59.0	89	65.4

tially circumcised, and 1 was uncircumcised—we have no details to explain the latter. To further confirm the apparent, more complete, circumcision status of Jewish males, 50 additional patients were examined at Cedars of Lebanon Hospital, with the results shown in table 8. Of the 6 partially circumcised, 5 were classed as grade 1 (nearly circumcised—*see* later).

When surgery is not performed, it would be worthwhile to estimate the extent to which nature itself partially endows the male with the anatomical features of circumcision. An estimate depends on what assumptions one is willing to make. Approximately one third of men who do not claim circumcision show partial lack of foreskin. Some of this may be due to wrong information about an operation. But the proportion with "partial circumcision" among younger men, age 40 to 59, who do not claim circumcision, is almost one third (31%); and the younger men may be able to answer the questions better. The comparable proportion for men age 60 and over is 37 percent. Furthermore, patients who say they were

not circumcised, and whose longest occupation was a white-collar job, show 32 percent partial circumcision, and manual workers and farmers, 38 percent partial circumcision. These 2 occupational groups show essentially no difference at any other point in the cross-classification of patient's answer by physician's examination. Among foreign-born men, 32 percent show partial circumcision, though this is for the total group regardless of the patient's report—there were too few (22) to cross-classify. Thus, the estimate of the proportion not having an operation but naturally showing some lack of foreskin may range between one fourth and one third. It is interesting that this is close to the estimate, based on men claiming circumcision, that one third of operations in non-Jewish men have an incomplete effect, indicating perhaps that nature is as effective as surgery in this proportion of cases. Some men with a naturally shortened foreskin may report themselves as circumcised, and some, of course, may actually have had an operation. We can make only rough approximations of estimates subject to later corrections if data become available.

The classification of partial circumcision seems to be a true intermediate grade rather than one only for the grading of doubtful diagnosis of circumcision. In the course of the survey the examining physicians felt it worthwhile to check this point by breaking the intermediate classification into 4 grades, *i.e.*, from nearly circumcised, grade 1, to almost uncircumcised, grade 4. This was done for 33 non-Jewish patients, with these results in grades 1 to 4: 5, 13, 10, and 5. This is a small number on which to draw a conclusion, and verification should be tested with different physicians as well as more subjects. We cannot, however, say that the proportions distributed over each of the 4 grades of partial circumcision agree completely between men who claim and men who do not claim circumcision. The few numbers, in fact, suggest that men who claim circumcision will not be found in grade 4 and men who say they are uncircumcised will not be found in grade 1, as one would expect. But roughly equal proportions of each group may be found in the intermediate range. The over-all proportions found partially circumcised are larger than those found in a survey of British Air Force recruits reported by Carne (*6*). In the routine examination of recruits age 17 to 24, Carne found 34.4 percent circumcised and 6.7 percent who had a foreskin covering half or less of the glans. Differences in definitions and in methods of conducting examinations could account for at least part of the discrepancy. In addition, circumcision in Great Britain may result in a more complete removal of foreskin.

For what it is worth, it is possible to make rough estimates of the accuracy of men's responses by referring to table 8. Assuming that about one third of circumcisions are partially rather than completely effective, 15 percent or less of those who say they are circumcised may be wrong. Of men who say they have not been circumcised, some 7 percent may be wrong. As table 8 shows, there appears to be an age difference in the agreement between patients and physicians. More disagreements are found among older men, but even here they occur more often among men

who claim to be circumcised than among those who say they are not circumcised.

In other examinations of data where occupation of father was available, we found a difference in partial circumcision between men born into a family in which the father was a manual worker or a farmer (57 out of 132) and those whose father was a white-collar worker (10 out of 41). We are not sure what, if any, significance this has. We did not, as we thought we might, find more uncircumcised in those who came from the family of a manual worker or a farmer. British data on Air Force recruits, age 17 to 24, presented by Carne, show more circumcision in those with better education (*6*). The present occupational data are highly limited because the source of data would restrict any differences.

Discussion

Disregarding the possibility that other factors in addition to circumcision might account for the advantage among Jewish women, the evidence suggests that a study of cervical cancer in non-Jewish women should disclose a relative risk of fivefold or more for those exposed to uncircumcised partners. As pointed out earlier, the study as conducted should have disclosed a relative risk of 2 or 2.5 to 1 or greater, but a relative risk of lower magnitude might have gone undetected. The limits of adequacy of our study may still have failed to reveal a true protection from cervical cancer resulting from male circumcision, comparable to that experienced by Jewish women, because of poor identification of completely circumcised non-Jewish men. The evidence gained from examining men shows, theoretically, how the actual risk ratio could be minimized below the level of sensitivity of our study.

We found that Jewish men seem to be fully circumcised, while only half of non-Jewish men who believe themselves to be circumcised actually lost all foreskin as a result of the operation. If the magnitude of protection of Jewish women is associated with having a partner who is fully circumcised, the relative risk could be minimized in an interview study of non-Jewish women which failed to identify partners with incomplete circumcision. We also found that some men may have a naturally shortened foreskin somewhat like the incompletely circumcised, and the inclusion of these men with the uncircumcised could further dilute the results if this status affords some intermediate degree of protection.

From the data in table 8, the diluting effect of the discrepancy between stated and anatomically judged circumcision can be seen readily. If anatomically complete circumcision is the only one that offers any protection to exposed women, and if any other circumcision status increased the cervical-cancer risk of exposed women fivefold, the demonstrated relative risk associated with uncircumcised males under the conditions of table 8 would be

$$\frac{(.066 \times 1) + (.934 \times 5)}{(.477 \times 1) + (.523 \times 5)} = \frac{4.736}{3.092} = 1.5.$$

If any degree of anatomical circumcision offered the stated amount of protection, the relative risk demonstrated to be associated with males stating they are uncircumcised would be

$$\frac{(.410 \times 1) + (.590 \times 5)}{(.863 \times 1) + (.136 \times 5)} = \frac{3.360}{1.543} = 2.2.$$

If an intermediate degree of protection were afforded by anatomically partial circumcision—perhaps 2.5-fold as compared to the anatomically complete circumcision—the demonstrated relative risk associated with the male who states he is uncircumcised would be

$$\frac{(.066 \times 1) + (.344 \times 2.5) + (.590 \times 5)}{(.477 \times 1) + (.386 \times 2.5) + (.136 \times 5)} = \frac{3.876}{2.122} = 1.8.$$

Further dilution would occur, of course, if one were to consider multiple partners.

If there are diluting effects due to misclassification when women rather than the men themselves are evaluating the circumcision status—and it is difficult to believe that this is not the case—it becomes unlikely that such studies as are being discussed here will demonstrate an advantage to women from exposure only to circumcised males. Certainly, demonstration of an advantage comparable to that experienced by Jewish women would not be expected. While the discrepancy of misclassification can dilute the relative risk of a pertinent variable to the point of nonrecognition, we do not agree that misclassification *per se*, as implied by Lilienfeld and Graham in their discussion, could give a spurious significance to an unrelated variable. This would only be true if there were a differential bias in misclassification between patients and controls.

It may be that the problem can be settled only in a study which makes direct examinations of sexual partners to distinguish full and partial circumcision. If the solution of the problem also depends on knowing circumcision status of partners in all sexual contacts, then one must also accept some verbal reports from women. Direct examinations, however, would be feasible on living husbands of women married once only. If a large number of these women admitted to only minimal or no nonmarital contacts, the concentration on these women may also be sufficient for the hypothesis. Since this group of women is a relatively low-risk group, more time and effort is necessary to accumulate cervical-cancer cases. To compensate for this it would be possible to observe complete circumcision. If complete circumcision reduces the risk of cervical cancer to as little as one fifth the rate experienced generally, a true advantage could be demonstrated with a fairly small sample.

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Further Observations on the Chromosomes of Chinese Hamster Cells in Tissue Culture[1,2]

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Summary

The chromosome complements of 3 Chinese hamster cell strains and a methylcholanthrene-treated subline varied during observation periods of 12 to 22 months. The complements were formed basically from multiples of the haploid constitution, and in 1 strain the normal diploid complement persisted as the predominant pattern for many months. Abnormalities of varying types began to appear soon after a culture was initiated, and in some sublines particular abnormal chromosome patterns persisted for many months, while in others variation was continuous. Except in the rare, markedly polyploid and partially disintegrated metaphases, the normal morphology of the individual chromosomes was usually maintained. Thus the metaphase chromosomes could nearly always be identified, though exact similarity to the normal types was not found when deletions resulted in partial loss of chromosome arms. Telocentric chromosomes, not present in the normal diploid complement, were a feature of most strains and in some cases could be related to centromeric misdivision with persistence of 1 or 2 arms as functioning chromosomes. Other aberrant chromosome forms could not be identified with normal chromosome types. The changes in chromosome complements in these nonclonal cultures were fluctuating events unassociated with obvious changes in cultural or morphological appearances. A characteristic chromosome pattern with an unusual chromosome form appeared in a methylcholanthrene-treated subline of 1 cell strain, 5 months after exposure to the carcinogen. Subsequently, however, this characteristic pattern disappeared, and the chromosome complement of the treated subline did not differ significantly from the untreated parent cell strain.—**J. Nat. Cancer Inst. 22: 765–799, 1959.**

Variations in the chromosome number of normal Chinese hamster cells grown in tissue culture for prolonged periods were studied (*1*, *2*). At the same time, an analysis of diploid metaphases for chromosome distribution and morphology revealed particular abnormalities in the chromosome

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complements. The investigation was continued and this report is concerned with further observations on the chromosome patterns in the cell strains, the study of metaphases with more than diploid numbers of chromosomes, the characterization of aberrations involving the various individual chromosome types, and the examination of cells in anaphase. The same methods and materials were used as previously (*1*), and observations were continued on cell strains V, P, C, and the methylcholanthrene-treated substrain C-M/C of cell strain C. As previously described, each of these 4 strains has been grown in duplicate as sublines 1 and 2 of the respective strain, and these separate sublines have had no contact with each other for the past year, each being treated as a distinct culture.

Results

Observations on Chromosome Complements of Cell Strains

Table 1 shows the continued observations on the chromosome number of the 4 cell strains V, P, C, and C-M/C and, as before, the metaphase populations of the cell strains were divided on the basis of chromosome estimates into "diploid," "triploid," "tetraploid," and "more than tetraploid" groups. On each occasion sublines 1 and 2 of these strains were estimated separately, but in the preparation of table 1, the estimates of sublines 1 and 2 have been combined when they did not significantly differ from each other. However, when there was a difference between the estimates of the sublines of a cell strain, the figures have been listed separately in the table. As described in the earlier report, metaphases were analyzed for chromosome distribution and aberrations by recognizing the individual chromosome types from their size and position of the centromere, and the same limitations prevailed that were noted previously.

Both sublines of cell-strain V remained predominantly "diploid" during the 292 days of observation (table 1), and the classical diploid complement was the most frequent chromosome pattern found in the metaphases of both sublines up to the 228th day of culture. On the 292d day the commonest pattern in the V-2 metaphases was still the normal diploid complement, but in the V-1 subline only a few metaphases were of this type. In both sublines the abnormalities encountered were small and variable and usually consisted of an extra chromosome, such as a small metacentric, subterminal or telocentric, or the absence of a chromosome, most often a #3, 4, or 8. Figure 1 illustrates a diploid metaphase with a classical chromosome complement in the 10th month after initiating the cell strain.

Subline 2 of the P strain remained predominantly "diploid" throughout the 427-day observation period, though a moderate and variable number of "triploid" and "tetraploid" cells was also present (table 1). During the latter 9 months the commonest metaphase type in this subline 2 continued to be the complement reported earlier and characterized by an absence of a #3, 4, or 8 chromosome and the addition of both an extra #10 or 11 chromosome and 1 telocentric, giving a total count of 23. Sub-

TABLE 1.—*Chromosome estimates of cell lines V, P, C, and C-M/C, based on 100 observed cells on each date*

Cell strain and subline	Date (*1957–58*)	After initiation of culture (*days*)	Metaphases "diploid" (*percent*)	Metaphases "triploid" (*percent*)	Metaphases "tetraploid" (*percent*)	Metaphases "more than tetraploid" (*percent*)	Metaphases not estimable* (*percent*)
V-	Sept. 23	11	90	0	3	0	7
1	Nov. 4	53	86	0	7	0	7
1	Dec. 13	92	76	3	9	2	10
2	Jan. 5	115	89	2	3	0	6
1 + 2	Feb. 7	148	88	0	6	0	6
1 + 2	Mar. 2	171	87	4	8	1	0
1 + 2	Apr. 28	228	79	6	11	4	0
1 + 2	July 1	292	86	3	11	0	0
P- 2	Oct. 31	175	83	2	11	4	0
1 + 2	Jan. 12	248	58	26	11	2	3
1 + 2	Mar. 14	309	69	12	16	3	0
1 + 2	May 9	365	69	9	18	4	0
1	July 7	424	39	41	15	5	0
2	July 10	427	68	14	14	4	0
C-1	Oct. 24	359	63	12	21	1	3
	Dec. 17	413	59	35	2	2	2
	Jan. 8	435	8	82	2	4	4
	May 5	552	79	11	6	4	0
	July 29	637	81	9	9	1	0
C-2	Nov. 1	367	57	31	9	3	0
	Dec. 30†	426	28	56	5	11	0
	May 4	551	13	68	4	15	0
	July 31	639	8	33	51	8	0
C-M/C-1 + 2	Nov. 19	172‡	35	47	6	11	1
2	Jan. 8	222‡	4	86	4	6	0
1	Mar. 1	274‡	13	67	10	10	0
1 + 2	May 22	356‡	11	64	6	19	0
1 + 2	July 25	420‡	14	78	3	5	0

*For the latter 6 months, cells which could not be estimated were excluded from the 100 cells observed.
†Figures calculated from an analysis of 18 cells.
‡Time from exposure of substrain to methylcholanthrene.

line 1 of the P strain maintained a "diploid" chromosome number for the 1st year in culture, but on the 424th day, 41 percent of the metaphases were "triploid," 15 percent "tetraploid," and only 39 percent "diploid." Detailed chromosome analysis of this subline showed a new complement on the 309th day and, although the dominant metaphase type had a count of 22, a variety of abnormalities was present; thus, one of the #1 chromosomes had a deletion of about half of 1 arm, one of the #2 chromosomes had a deletion of about three fourths of 1 arm, one of the medium-sized metacentric chromosomes was usually absent, and an extra subterminal chromosome was usually present. The abnormalities in the #1 and 2 chromosomes are discussed more fully later, and a metaphase of this aberrant type is illustrated in figure 2. On the 424th and 443d days this pattern was no longer clearly evident and the metaphase population had no recognizable "stem line," but a variety of hyperdiploid and hypertriploid metaphases was seen, showing mixtures of the abnormalities observed in the other cell strains and described elsewhere in this report.

Subline 1 of strain C, which had remained predominantly "diploid" for the 1st year in culture, gradually showed an increasing number of "triploid" metaphases, up to 35 percent on the 413th day (table 1). Chromosome analysis of "diploid" metaphases on this day revealed the commonest pattern to be a metaphase characterized by 24 chromosomes—the normal complement with an additional small metacentric #6 or 7 and 1 telocentric. This complement has already been described and illustrated with photographs in the previous report (*1*). No analysis of polyploid metaphases was made until January 8, 1958, the 435th day, when over 80 percent of the metaphases in the subline were "triploid" on estimations of the chromosome number. These metaphases, on analysis, were found to be hypertriploid, and a description of their characteristics is given later in the account of the observations on polyploid cells. Unfortunately, all the C-strain sublines grew very poorly during February, 1958, and no further examination of subline 1 was made until May, 1958, when the culture was discovered to be predominantly "diploid." This reversal from a hypertriploid state to a "diploid" was surprising, and raised once again the question of laboratory error from cell cross-contamination. However, chromosome analysis on the 551st day showed that the commonest pattern was the same as when "diploids" had previously been dominant and which has been described earlier; moreover, this pattern was not characteristic of any other subline. Reexamination on the 639th day revealed a persistence of this near-diploid pattern both on chromosome number estimates and on detailed analysis.

Subline 2 of the C strain behaved differently. Like subline 1, the "diploids" were replaced by "triploid" cells between the 14th to 19th month of culture but no reversal to diploidy was observed. On the contrary, the chromosome number became further elevated by the 639th day, when metaphases with 40 to 46 chromosomes became predominant. Chromosome analysis on the 551st day showed that the hypertriploid metaphases were characterized by several aberrant chromosomes (fig. 3).

One of the #1 chromosomes had a deletion of about half of 1 arm and one of the #2 chromosomes had a deletion of about three fourths of 1 arm; both of these abnormalities have already been noted as occurring in "diploid" metaphases of subline 1 of the P strain. In addition, a large subterminal chromosome with a body length approximately equal to the long arm of a #1 chromosome was present and resembled closely the large subterminal chromosome previously reported in the hypodiploid metaphases of the C-M/C substrain. However, this new chromosome form in the C strain, subline 2, was characterized by a marked secondary constriction in the mid-body region, whereas this was not evident in the C-M/C chromosome. On the 639th day, analysis of the hypotetraploid and tetraploid metaphases showed that some chromosome types were represented according to triploid values and others according to tetraploid values; a moderate number of the metaphases had large subterminal chromosomes, though the characteristic pattern of these was not as marked as 2 months previously. This state of hypotetraploidy has not been a marked feature of any other subline, but in all "triploid" C sublines there has always been about 5 percent of metaphases with chromosome counts of 28 to 32 in which some chromosome types are represented according to diploid values and others according to the number expected in triploid cells.

At the time of the last report the majority of metaphases in the C-M/C substrain had become "triploid," but a selective analysis of the "diploid" metaphases showed a hypodiploid complement characterized by a chromosome count of 21, an absence of one #3, 4, or 8 chromosome and one #6 or 7 chromosome, a deletion of half of 1 arm of a #1 chromosome, and a new large subterminal chromosome form. During the succeeding 9 months, both sublines continued to be predominantly "triploid" (table 1), and no significant differences were observed between sublines 1 and 2 of this C-M/C substrain either on chromosome estimates or chromosome analysis. A detailed study of the "triploid" C-M/C-2 metaphases was made on January 8, 1958, in the 8th month after methylcholanthrene treatment, and the results are given later in the description of the observations on polyploid cells. No significant difference could be demonstrated between these hypertriploid metaphases and those of C, subline 1. In general, the complements were basically triploid with the addition of extra #6 or 7, extra #9 to 11, and telocentric chromosomes. At this time of sampling, the characteristic hypodiploid metaphases observed 3 months before were no longer evident and the large subterminal chromosome was rarely seen. From January to July, 1958, the chromosome complements of both sublines did not differ fundamentally from the patterns observed on January 8th.

Observations on Polyploid Cells

Metaphases with more than diploid numbers of chromosomes were studied in cell strain C, subline 1, 15 months after initiation of the culture, and in subline 2 of the methylcholanthrene-treated C-M/C substrain, 7

months after exposure to the carcinogen. As already noted, both the sublines were predominantly in the triploid range (82% and 86%, respectively, table 1) when the observations were made, though small numbers of "diploid," "tetraploid," and "more than tetraploid" metaphases were also encountered. Seventy metaphases with less than 41 chromosomes were analyzed in each subline and it was possible to achieve almost the same degree of accuracy, in classifying the individual types, which was attained during the study of diploid metaphases. The #1 and 2 chromosomes could be individually recognized but the remainder were only differentiated into 4 groups: medium-sized metacentric chromosomes #3, 4, and 8; small metacentric chromosomes #5, 6, and 7; subterminal chromosomes #9, 10, and 11; and telocentric chromosomes. After each metaphase was drawn and analyzed the complement was evaluated to judge if it was basically diploid, triploid, or not clearly related to either haploid multiple, "n." In tables 2 and 3, which give the results of these analyses, the degree of ploidy "n" is presented in column 2. Metaphases with 21 to 26 chromosomes were considered to be basically diploid, and those with 32 to 38 chromosomes basically triploid. This subjective procedure for categorizing metaphases with tetraploid or less numbers of chromosomes is meaningful in Chinese hamster cells in which the chromosome types of the haploid complement are distinguishable; this is in contrast to the study of chromosomes in mouse cells in which mathematical criteria only can be used to determine the degree of ploidy. The great majority of the 70 metaphases of each subline could be regarded as basically diploid or triploid and, in these cases, it was possible to calculate the number of chromosomes that would have been expected in each chromosome group if the metaphases had been classically diploid or triploid. The difference between the expected number and the number actually observed in each group gave the $\pm$ variation from the expected number, and this figure has been included in the tables.

Table 4 summarizes the data from tables 2 and 3 and compares the results obtained in the 2 sublines. The table shows that 62 of the 70 C and 58 of the 70 C-M/C metaphases were "triploid." The majority of each subline, however, were hypertriploid, with chromosome counts of 34 to 38, which was increased above the classical triploid complement because of 1 or 2 extra #5, 6, or 7 chromosomes, 1 or 2 extra #9, 10, or 11 chromosomes, and 1 to 3 telocentric chromosomes. In a considerable number of metaphases there was a shortage of #3, 4, or 8 chromosomes, though this was not as marked as the increase in the 5-to-7 and 9-to-11 groups. Thus, in the 62 C, subline 1, metaphases considered to be basically triploid, there were 50 less #3, 4, or 8 chromosomes than were expected, in contrast to an excess of 65 and 58 chromosomes in the 5-to-7 and 9-to-11 groups, respectively. Likewise, in the C-M/C, subline 2, 58 metaphases considered to be basically triploid had 21 less #3, 4, or 8 chromosomes and 51 excess in both the 5-to-7 and 9-to-11 groups. The observed numbers of #1 and 2 chromosomes were also reduced somewhat below the expected numbers.

In tables 2 and 3 the group of #1 chromosomes is marked with an asterisk when one of the chromosomes had a deletion of about one half of 1 arm, a relatively common event occurring in 52 C-1 and 38 C-M/C-2 subline metaphases. The appearances of these hypertriploid metaphases are illustrated in figures 5 and 6.

At this time of sampling, the 2 sublines could not be distinguished by a morphological examination of their chromosome complements, and table 4 shows a comparison of the findings in the 70 metaphases of both cell lines. The chi-square test was applied with the hypothesis that the number of chromosomes of each type was represented proportionately to the total number of chromosomes observed in the 70 cells of each subline. No statistically significant difference was found in the frequency with which the chromosome types of the 2 sublines were observed. Thus, it was not possible to demonstrate that methylcholanthrene treatment had caused any morphological changes in the 7 months following exposure to the carcinogen.

In addition to the observations already described, metaphases with tetraploid or greater numbers of chromosomes, forming 4 percent and 6 percent of the respective populations (table 1), were selected for analysis, and 23 C-1 and 16 C-M/C-2 subline metaphases were examined, with the results shown in table 5. Analysis of these more highly polyploid metaphases did not allow adequate differentiation between the #1 and 2 chromosomes, and the complements have thus been differentiated into 5 groups only. Also, in highly polyploid metaphases it is not possible to determine with accuracy the probable degree of ploidy and, therefore, the number of chromosomes in each group has been expressed both as an absolute number and as a percentage of the total number in the metaphase. It is seen from the table that the pattern is relatively constant throughout the wide range of chromosome counts and this indicates that the complements are based on multiples of the haploid constitution. As already observed in "diploid" and "triploid" metaphases, there is a relative deficiency of #3, 4, and 8 chromosomes, and this consistent loss of medium-sized metacentric chromosomes is believed to support the morphological evidence that the telocentric chromosomes are in some cases derived from centric fracture of one of the chromosomes in this group. Recognition of the individual chromosome types in highly polyploid cells is quite feasible as illustrated in figure 7.

Observations on Chromosome Morphology

In this and in the previous reports various aberrations from the normal chromosome morphology have been mentioned but not discussed in detail. In general, when the degree of ploidy was tetraploid or less, the structural abnormalities were restricted in number and were repeated in definite patterns in successive subcultures which indicated their persistence through a number of cell cycles. This was in contrast to the highly polyploid and partially disintegrated metaphases encountered rarely in all strains in which chromosome aberrations of all possible types were

TABLE 2.—*Cell strain C, subline 1, January 8, 1958, 15th month of culture* in vitro; *chromosome complement of 70 metaphases with less than tetraploid numbers of chromosomes*

Chromosome count (*total elements*)	Basic degree of ploidy "n"	Chromosome #1		Chromosome #2		Chromosomes #3, 4, and 8		Chromosomes #5, 6, and 7		Chromosomes #9, 10, and 11		Telocentric chromosomes
		Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number
24	2	2		1	−1	7	+1	6		6		2
24†	2	2		2		5	−1	8	+2	6		1
25	2	2		2		7		6		6		2
30	?	3	?	3	?	6	?	8	?	9	?	1
31†	?	1	?	2	?	7	?	10	?	8	?	2
32	3	3*		3		7	−2	9		9		1
32	3	3*		2	−1	7	−2	8	−1	9		3
32	3	3*		3		6	−3	9		10	+1	1
33	3	3		3		7	−2	8	−1	11	+2	1
33	3	3*		3		7	−2	9		9		2
33	3	2	−1	2	−1	8	−1	9		9		3
33	3	2	−1	2	−1	7	−2	10	+1	10	+1	2
33	3	3		2	−1	7	−2	10	+1	8	−1	3
34	3	3*		3		8	−1	10	+1	9		1

35	3	3		3		8	−1	11	+2	9		1
35	3	3		3		8	−1	10	+1	10	+1	1
35	3	3*		3		8	−1	9		10	+1	2
35†	3	4*	+1	3		8	−1	9		10	+1	1
35	3	3*		3		9		8	−1	10	+1	2
35	3	3*		3		8	−1	10	+1	9		2
35	3	3*		3		8	−1	10	+1	10	+1	1
35	3	3*		3		7	−2	11	+2	10	+1	1
35	3	3*		2	−1	9		10	+1	10	+1	1
35	3	3		3		9		9		10	+1	1
36	3	2	−1	3		8	−1	11	+2	10	+1	2
36	3	4*	+1	2	−1	8	−1	10	+1	10	+1	2
36	3	3*		3		9		9		10	+1	2
36	3	3*		3		8	−1	11	+2	9		2
36	3	3*		3		8	−1	10	+1	11	+2	1
36	3	3*		3		7	−2	10	+1	11	+2	2
36	3	3*		3		8	−1	11	+2	10	+1	1
36	3	3*		3		9		10	+1	9		2
36	3	3		3		9		10	+1	10	+1	1
36	3	3*		3		9		10	+1	10	+1	1

See footnotes at end of table.

TABLE 2.—*Cell strain C, subline 1, January 8, 1958, 15th month of culture* in vitro; *chromosome complement of 70 metaphases with less than tetraploid numbers of chromosomes*—Continued

Chromosome count (*total elements*)	Basic degree of ploidy "n"	Chromosome #1		Chromosome #2		Chromosomes #3, 4, and 8		Chromosomes #5, 6, and 7		Chromosomes #9, 10, and 11		Telocentric chromosomes
		Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number
36	3	3*		3		9		10	+1	10	+1	1
36	3	3*		3		9		10	+1	10	+1	1
36	3	3*		3		8	−1	11	+2	10	+1	1
36	3	3*		3		8	−1	11	+2	9		2
36	3	3*		2	−1	9		10	+1	11	+2	1
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		8	−1	10	+1	10	+1	3
37	3	3*		4	+1	8	−1	10	+1	10	+1	2
37	3	3		3		8	−1	11	+2	10	+1	2
37	3	3*		3		8	−1	11	+2	10	+1	2
37	3	3		3		8	−1	10	+1	10	+1	3
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		9		10	+1	3

37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		8	−1	11	+2	10	+1	2
37	3	3		3		9		10	+1	10	+1	2
37	3	3*		3		7	−2	11	+2	10	+1	3
37	3	3*		3		7	−2	11	+2	10	+1	3
37	3	3*		3		7	−2	12	+3	10	+1	2
37	3	3*		3		9		9		11	+2	2
37	3	3*		3		8	−1	11	+2	10	+1	2
37†	3	3*		3		8	−1	10	+1	11	+2	2
38	3	3*		3		9		10	+1	10	+1	3
38	3	3*		3		8	−1	11	+2	10	+1	3
38	3	2*	−1	3		8	−1	11	+2	11	+2	3
38	3	2	−1	3		9		11	+2	10	+1	3
38	3	3*		3		9		10	+1	10	+1	3
38	3	3*		3		9		10	+1	10	+1	3
38	3	3*		3		9		11	+2	10	+1	2
38	3	3*		3		9		10	+1	11	+2	2
39	?	3*	?	3	?	10	?	11	?	10	?	2
39	?	3*	?	3	?	9	?	12	?	10	?	2
40	?	3*	?	3	?	9	?	11	?	11	?	3

*Indicates one of the #1 chromosomes showed a deletion of half of 1 arm.
†Indicates presence of a dicentric chromosome or minute fragments.

TABLE 3.—*Cell strain C-M/C, subline 2, January 8, 1958, 7 months after methylcholanthrene treatment; chromosome complement of 70 metaphases with less than tetraploid numbers of chromosomes*

Chromosome count (*total elements*)	Basic degree of ploidy "n"	Chromosome #1		Chromosome #2		Chromosomes #3, 4, and 8		Chromosomes #5, 6, and 7		Chromosomes #9, 10, and 11		Telocentric chromosomes
		Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number
21	2	2		2		5	−1	6		6		0
24	2	2*		2		5	−1	6		7	+1	2
24	2	1	−1	5	+3	5	−1	6		6		1
27	?	2	?	2	?	5	?	7	?	7	?	4
28	?	1	?	2	?	8	?	7	?	9	?	1
28	?	2	?	2	?	7	?	8	?	7	?	2
29	?	2	?	2	?	7	?	9	?	8	?	1
29	?	2	?	3	?	6	?	8	?	9	?	1
30	?	2*	?	3	?	6	?	8	?	9	?	2
31†	?	2	?	3	?	8	?	7	?	9	?	1
32	3	2*	−1	3		8	−1	9		9		1
32	3	2	−1	2	−1	8	−1	9		10	+1	1
33	3	2	−1	3		9		9		8	−1	2
34	3	3		3		9		9		9		1
34	3	3*		3		8	−1	10	+1	9		1

34	3	3*		3		8	−1	10	+1	9		1
34	3	3*		3		7	−2	9		10	+1	2
34	3	3*		3		8	−1	9		10	+1	1
34	3	3*		3		8	−1	9		10	+1	1
34	3	3*		3		9		9		9		1
35†	3	3		3		8	−1	9		9		2
35	3	3*		2	−1	8	−1	10	+1	11	+2	1
35	3	3*		3		9		10	+1	9		1
35	3	3*		3		9		9		10	+1	1
35	3	3		3		9		10	+1	9		1
35	3	3*		3		9		9		10	+1	1
35	3	3*		3		9		9		9		2
35	3	3		3		9		10	+1	9		1
35	3	3*		3		9		10	+1	9		1
35	3	3		3		9		10	+1	9		1
35	3	3*		3		9		8	−1	11	+2	1
35	3	2	−1	3		9		9		11	+2	1
36	3	3		3		9		10	+1	9		2
36	3	3		3		9		11	+2	9		1
36	3	3		3		9		10	+1	10	+1	1

See footnotes at end of table.

TABLE 3.—*Cell strain C-M/C, subline 2, January 8, 1958, 7 months after methylcholanthrene treatment; chromosome complement of 70 metaphases with less than tetraploid numbers of chromosomes*—Continued

Chromosome count (*total elements*)	Basic degree of ploidy "n"	Chromosome #1		Chromosome #2		Chromosomes #3, 4, and 8		Chromosomes #5, 6, and 7		Chromosomes #9, 10, and 11		Telocentric chromosomes
		Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number	Variation from expected number	Number
36	3	3		3		9		10	+1	10	+1	1
36	3	3*		3		8	−1	10	+1	11	+2	1
36	3	3		3		9		10	+1	10	+1	1
36	3	3		3		9		11	+2	10	+1	0
36	3	3*		3		8	−1	10	+1	10	+1	2
36	3	3		3		9		10	+1	10	+1	1
36	3	3*		3		9		10	+1	9		2
36	3	2*	−1	2	−1	9		10	+1	11	+2	2
36	3	3*		3		8	−1	10	+1	10	+1	2
36	3	3*		3		8	−1	10	+1	10	+1	2
36	3	3*		3		8	−1	10	+1	10	+1	2
36†	3	1	−2	5	+2	8	−1	10	+1	10	+1	2
36	3	3*		3		8	−1	10	+1	10	+1	2
36	3	3		3		8	−1	11	+2	9		2
36	3	3*		3		8	−1	11	+2	10	+1	1
36	3	3		3		9		10	+1	10	+1	1

36	3	3*		3		9		9		11	+2	1
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		10	+1	10	+1	2
37	3	3*		3		9		10	+1	10	+1	2
37	3	3		3		9		10	+1	10	+1	2
37	3	3*		3		8	−1	11	+2	10	+1	2
37	3	3*		3		8	−1	11	+2	10	+1	2
37	3	3*		3		9		11	+2	10	+1	1
38	3	3		3		8	−1	10	+1	11	+2	3
38	3	3*		3		9		10	+1	10	+1	3
38	3	3		3		9		10	+1	12	+3	1
38	3	3*		3		10	+1	10	+1	10	+1	2
38	3	3		3		10	+1	10	+1	10	+1	2
38	3	3		3		9		11	+2	10	+1	2
38	3	3*		3		8	−1	11	+2	11	+2	2
38	3	3*		3		9		10	+1	11	+2	2
38	3	3*		3		9		10	+1	10	+1	3
40	?	3*	?	3	?	10	?	11	?	11	?	2
41	?	3	?	4	?	9	?	11	?	11	?	3

* Indicates one of the #1 chromosomes showed deletion of half of 1 arm.
† Indicates presence of a dicentric chromosome or minute fragments.

TABLE 4.—*Comparison of data from tables 2 and 3*

Cell strain		Chromosome types						Total chromosomes
		#1	#2	#3, 4, 8	#5, 6, 7	#9, 10, 11	Telocentric	
62 "Triploid" C metaphases	Observed number	183	180	508	623	616	119	2229
	Expected number	186	186	558	558	558	0	2046
	Difference	−3	−6	−50	+65	+58	119	+183
58 "Triploid" C-M/C metaphases	Observed number	167	173	501	573	573	88	2075
	Expected number	174	174	522	522	522	0	1914
	Difference	−7	−1	−21	+51	+51	88	+161
70 C 70 C-M/C metaphases	Observed number	201	199	568	695	682	134	2479
	Observed number	192	216	582	667	672	108	2437
	X^2	0. 06	1. 0	0. 4	0. 3	0. 01	2. 4	
	P	0. 8	0. 3	0. 5	0. 6	0. 9	0. 13	

TABLE 5.—*Chromosome complements of metaphases with tetraploid, or greater, numbers of chromosomes arranged in order of increasing chromosome number*

Cell line	Chromosome count (*total elements*)	Large metacentric chromosomes #1 and 2		Medium metacentric chromosomes #3, 4, and 8		Small metacentric chromosomes #5, 6, and 7		Subterminal chromosomes #9, 10, and 11		Telocentric chromosomes	
		Number	Percent of total (*normally 18.1*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 0*)
	45	8	17.8	10	22.2	14	31.2	11	24.2	2	4.4
	45	8	17.8	12	26.7	12	26.7	11	24.4	2	4.4
	50	8	16.0	12	24.0	15	30.0	13	26.0	2	4.0
	52	10	19.2	13	25.0	13	25.0	14	26.9	2	3.9
	53	11	20.8	7	13.2	13	24.5	17	32.1	5	9.4
	53	9	16.9	7	13.2	17	32.0	17	32.0	2	3.7
	55	8	14.5	14	25.5	14	25.5	15	27.3	4	7.3
	57	10	17.5	15	26.3	15	26.3	15	26.3	2	3.5
	57	10	17.5	15	26.3	15	26.3	17	29.8	2	3.7
	59	10	16.9	13	22.0	16	27.1	16	27.1	4	6.7
	60	9	15.0	13	21.7	18	30.0	18	30.0	2	3.3
	64	10	15.6	16	25.0	15	23.4	18	28.2	5	7.8
	68	10	14.7	19	27.9	20	29.4	17	25.0	2	3.0

TABLE 5.—*Chromosome complements of metaphases with tetraploid, or greater, numbers of chromosomes arranged in order of increasing chromosome number*—Continued

Cell line	Chromosome count (*total elements*)	Large metacentric chromosomes #1 and 2		Medium metacentric chromosomes #3, 4, and 8		Small metacentric chromosomes #5, 6 and 7		Subterminal chromosomes #9, 10, and 11		Telocentric chromosomes	
		Number	Percent of total (*normally 18.1*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 27.3*)	Number	Percent of total (*normally 0*)
C-1 subline, Jan. 8, 1958	69	12	17. 4	16	23. 2	18	26. 1	20	29. 0	3	4. 3
	69	9	13. 0	18	26. 1	20	30. 0	19	27. 5	3	4. 4
	70	12	17. 2	15	21. 4	22	31. 4	18	25. 7	3	4. 3
	71	10	14. 1	16	22. 5	20	28. 2	19	26. 8	6	8. 4
	71	10	14. 0	19	26. 7	21	29. 7	16	22. 6	5	7. 0
	71	11	15. 5	18	25. 4	20	28. 2	18	25. 4	4	5. 5
	72	12	16. 6	17	23. 6	22	30. 6	17	23. 6	4	5. 6
	75	12	16. 0	18	24. 0	22	29. 3	18	24. 0	5	6. 7
	89	13	14. 6	17	19. 1	32	36. 0	19	21. 3	6	6. 7
	118	18	15. 3	26	22. 0	38	32. 2	31	26. 3	5	4. 2
	1493	*240*	*16. 1*	*346*	*23. 2*	*434*	*29. 1*	*394*	*26. 4*	*80*	*5. 3*
	50	8	16. 0	10	20. 0	14	28. 0	15	30. 0	3	6. 0
	56	8	14. 3	13	23. 2	18	32. 1	15	26. 8	2	3. 6

C-M/C-2 subline, Jan. 8, 1958	58	6	10.3	17	29.3	14	24.1	17	29.3	4	7.0
	58	8	13.8	14	24.1	17	29.3	16	27.6	3	5.2
	62	8	12.9	16	25.8	16	25.8	18	29.0	4	6.5
	64	10	15.6	13	20.3	18	28.1	20	31.2	3	4.6
	67	10	14.9	16	23.9	22	32.8	17	25.4	2	3.0
	70	10	14.3	17	24.3	19	27.1	22	31.4	2	2.9
	72	10	13.8	17	23.6	22	30.5	20	27.7	3	4.1
	73	12	16.4	16	21.9	19	26.0	23	31.5	3	4.2
	74	12	16.2	18	24.3	20	27.0	22	29.7	2	2.8
	74	12	16.2	15	20.2	21	28.3	21	28.3	5	6.7
	77	14	18.2	17	22.1	21	27.2	22	28.6	3	3.9
	85	13	15.2	18	21.1	26	30.5	24	28.2	4	4.7
	114	18	15.7	27	23.6	30	26.3	30	26.3	9	7.8
	116	17	14.6	27	23.2	30	25.8	31	26.7	11	9.4
	1170	*176*	*15.0*	*271*	*23.2*	*327*	*27.9*	*333*	*28.5*	*63*	*5.4*

seen. Table 6 lists the more common abnormalities arising from the recognized chromosome types and also the more common aberrant chromosome forms, the origins of which were not clear. In addition to these abnormalities, each of the recognized chromosomes in occasional metaphases showed minor aberrations which resulted in small changes in their size and arm lengths but did not prevent the identification of the individual chromosome. Up to the time of this report, therefore, the great majority of chromosome forms seen in the metaphases were recognizable and closely related to their counterparts in the classical Chinese hamster metaphase; however, a study of the photographs of polyploid cells (figs. 3, and 5 through 8) shows that not every chromosome is clearly identifiable and that an occasional aberrant one is included within a given group on somewhat tenuous evidence.

In each cell strain and subline, one of the #1 chromosomes has shown, either in occasional metaphases or in many metaphases of successive subcultures, a deletion of about half of 1 arm, to give a chromosome with an arm ratio of 1:2.5 (figs. 2 and 3). These forms have been found in "diploid," "triploid," or more markedly polyploid metaphases, and sometimes intact #1 chromosomes have shown break-lesions at the site of this deletion. In the "diploid" P-1 subline in the 10th month of culture and in the hypertriploid C-2 subline in the 18th month, a deletion of about three fourths of 1 arm of one of the #2 chromosomes occurred, giving rise to the subterminal chromosome illustrated in figures 2 and 3. In addition to these identifiable deletions in chromosomes #1 and 2, the group of medium-sized metacentric chromosomes #3, 4, and 8 often showed variable small deletions in one or both arms, which were too inconsistent to define clearly and which gave rise to chromosomes of about the size of a #4 but with arm ratios in the region of 1:2. There is no evidence yet to suggest that the extra small metacentric and subterminal chromosomes frequently seen in the cultures could have arisen from deletions in the medium-sized metacentric chromosomes, but this possibility cannot be excluded. Apart from actual deletions, break-lesions were uncommonly observed in one or both chromatids, some resembling the secondary constriction often noted on the mid-distal portion of the long arm of normal #8 chromosomes.

As already observed in this and the previous report, metaphases often contained telocentric chromosomes of varying sizes which are not normally found in the classical complement of Chinese hamster cells. It has been suggested that these chromosomes in some instances may have resulted from centromeric fracture of medium-sized metacentric chromosomes with the persistence of the 2 halves as functioning units. This view has arisen from the observations that the appearance of 2 telocentric chromosomes often coincided with the disappearance of a #3 or 8 chromosome and that the lengths of the telocentrics were equal to the arm lengths of the absent chromosome (fig. 8). The absence of a medium-sized metacentric chromosome and the appearance of 1 or 2 telocentric chromosomes were probably the earliest and most frequent abnormalities encountered in these cell strains. However, telocentric chromosomes were

TABLE 6.—*Morphological aberrations observed in chromosomes of V, P, C, and C-M/C cell strains*

Chromosome	Nature of aberration	New chromosome form	Cell strains showing abnormality	Frequency in cell strain	Illustrated in figure
1	Deletion of about one half of 1 arm	Large chromosome with arm ratio of 1:2.5	C C-M/C P V	Common	2 and 3
2	Deletion of about three fourths of 1 arm	Large subterminal chromosome	P-1 C-2	Uncommon	2 and 3
3 and 8	Centromeric fracture	Two medium-sized telocentric chromosomes	C C-M/C P V	Common	8
3, 4, 8	Various deletions of one or both arms	Medium to small chromosomes with arm ratios of 1:2–1:3	C C-M/C P V	Common	
	?	Telocentric chromosomes	C C-M/C P V	Common	3, 5, 6 7, 8, 9
	?	Large subterminal chromosome with body length of long arm #1	C-M/C	Uncommon	4
	?	Large subterminal chromosome with constriction in mid-part of body	C-2	Uncommon	3
	?	Fragments including dot deletions	C C-M/C P V	Uncommon	
	?	Dicentric chromosomes of varying sizes	C C-M/C P	Rare	9
	?	Chromosome resembling large #9	C	Rare	
	?	Chromosome resembling small #11	C-M/C	Rare	
	?	Ring chromosomes	C C-M/C	Very rare	9

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also found when the complement of #3, 4, and 8 metacentric chromosomes was normal. Moreover, the lengths of the telocentric chromosomes were not always the same as the arm lengths of the metacentric chromosomes. Thus, other methods of origin must have existed and, although centromeric fracture of #1 and 9 chromosome has been observed, in many cases the origin of the telocentric is not known.

Unusual, large, subterminal chromosomes with a body size approximating the long arm of a #1 chromosome were seen rarely in all cultures, but on two occasions they formed a characteristic feature of dominant patterns. In the C-M/C cultures during the 5th and 6th months following methylcholanthrene treatment, a hypodiploid complement contained one of these large, subterminal chromosomes (fig. 4). In the 18th month of culture the C-2 subline showed a superficially similar large subterminal chromosome in hypertriploid metaphases, but on this occasion the body of the chromosome usually demonstrated a constriction in the middle of the long arm (fig. 3). In both these complements the appearance of the subterminal chromosome could have been related to the absence of a #3 or 8, but no evidence supporting any mechanism of transformation was available. Dicentric chromosomes of varying sizes were encountered sporadically and usually in polyploid cells (fig. 9); occasionally these dicentrics were "equal-armed," suggesting their origin from isolocus breaks in a chromosome following sister reunion and nondisjunction. A variety of new chromosome forms were occasionally seen and these included ring chromosomes and forms resembling abnormally large #9's or unusually small 11's. Highly polyploid metaphases revealed aberrations with more bizarre types; polycentric and acentric chromosomes, chromosome and chromatid interchanges (triradials and quadriradials, fig. 10), and minutes were seen in these severely damaged metaphases. In several highly polyploid metaphases with counts of about 100 chromosomes, unusual forms were found in which one long arm showed thin, folded chromatids connected with transverse connections, while others had elongated centromeric regions and fine chromatids.

Observations on Cells in Anaphase

At the times the observations were made, anaphases with structural abnormalities were scarce in the predominantly "triploid" C and C-M/C sublines and were more common in the "diploid" cells of the V and P sublines. The most frequently observed aberrations among anaphases were the single bridge with a single fragment, as seen in figure 11, and the single fragment alone illustrated in the telophase of figure 12. The majority of bridges and fragments were derived from chromosome or isochromatid breaks followed by sister-reunion of the broken chromatid ends, as indicated by equal-armed bridges in anaphases and equal-armed dicentrics in colchicine-treated cells. Sometimes sister-reunion of the centric portion was incomplete as shown in figure 13. In a few instances the bridge was observed without an accompanying fragment, which suggested the breakage-fusion-bridge cycle; sporadically the "short-armed"

#1 chromosomes showed a delayed orientation on the metaphase plate (fig. 16).

Polyploid cells exhibited single and double crisscross bridges involving subterminal and metacentric chromosomes (fig. 14), and these bridges were also seen in cells with multipolar spindles. Some highly polyploid mitoses showed excessive chromosome stickiness (fig. 15), while others showed varying degrees of chromosome fragmentation and disintegration. In dividing cells, with moderately disintegrated chromosomes (fig. 16), scattered acentric fragments could be seen when the centric portions of the chromosomes migrated toward the metaphase plates, but severe chromosome disintegration probably prevented the completion of mitosis to form 2 daughter nuclei. The reconstitution of single abnormal nuclei in these cells was associated with irregular dispersed chromatin throughout the entire cytoplasm, as was revealed by Feulgen staining of P and V cultures.

Discussion

One and a half years' observation on the chromosomes of Chinese hamster cells grown in tissue culture has not revealed any constant uniform behavior, but certain trends are apparent. In particular, states of hyperdiploidy and hypertriploidy are commonly found in which the increase in chromosome number above exact diploidy or triploidy results from an excess of certain chromosomes. On the other hand, it is remarkable how long the V strain has maintained the classical diploid pattern or a complement very close to it. As noted in the previous report, few serial observations on the chromosomes of cell strains in tissue culture have been published, and there is little available data to compare with the results obtained in these strains. Tjio and Puck (*3*) observed the chromosomes of several human, opossum, and Chinese hamster cell strains over periods of months and found that the cells of the former two species maintained their normal diploid state, whereas the Chinese hamster cells showed variations in chromosome number. Hsu, Pomerat, and Moorhead (*4*), however, made serial counts on a human fibroblast strain and reported an early change to the heteroploid state. Levan and Biesele (*5*) made repeated observations on successive transplant generations of mouse cell cultures derived from a variety of tissues. Established cell lines, both of normal and of cancerous origin, were found to have similar chromosome behavior characterized by an unstable mitotic mechanism with variation in chromosome number and structure so that the stemline chromosome sets of the cultured cell strains differed from the normal chromosome complement of the species. The workers also noted that treatment with trypsin appeared to increase the incidence of chromosome breaks. The morphological and chromosomal cell alteration previously observed (*1*) in the Chinese hamster A strain, and thought probably to have been due to a human cell cross-contamination, is now definitely considered to have been an artifact. This conforms with the experience of other workers (*6*), and it is very possible that many of the

sudden dramatic changes in cell morphology and cultural behavior reported in tissue culture may have resulted from similar cell cross-contaminations. It is expected that clonal sublines of the V, P, and C strains would yield more uniform chromosome complements (*7*), and the cloning of these cells strains is a logical step in their subsequent investigation.

The cell strains under study have shown a variable rate of growth and occasionally have ceased to proliferate and have become degenerate for several weeks at a time. In the C sublines this was particularly evident in February, 1957, August, 1957, and February, 1958; in each case, however, spontaneous recovery occurred, except in subline 2 of strain C during August, 1957, which slowly died so that a new subline 2 had to be restarted from subline 1. These fluctuations in growth rate have been less marked in the P and V strains. No cause for their occurrence has been found with certainty, though serum toxicity is probably a factor (*8*). Since May, 1958, the calf serum has been heated to 56° C. for 30 minutes and the cultures have subsequently maintained a more uniformly rapid rate of growth with a lessened number of granular cells. During February, 1958, when rigorous sterility tests were performed to explain the unhealthy state of the C cultures, it was discovered that all the C sublines contained living pleuropneumonia-like organisms. The origin of these organisms is obscure but their presence seems to be innocuous, and it would appear that pleuropneumonia-like organisms are symbiotic contaminants of many tissue-culture cell strains (*9*).

A number of the spontaneous aberrations described have also been observed in several induced and spontaneous fibrosarcomas and adenocarcinomas propagated *in vivo* (*10*). Moreover, many chromosome deletions in cultured normal fibroblasts have similar sites of breakage to those found in cells of neoplasms transplantable *in vivo*. Thus, otherwise lethal deletions in experimentally propagated cells have been induced and have persisted both *in vitro* and *in vivo*. Competition among the more viable "lethals" is either slight or buffered by the accompanying increase in chromosome number. Exceptional mutations, such as those from centromeric fractures which give rise to telocentrics, may survive indefinitely and are, therefore, equivalent to "marker" chromosomes noted in experimental tissues of other species. The origin of telocentrics from centromeric misdivision is considered, by a number of cytologists, as the reverse of the origin of metacentric chromosomes by fusion of telocentrics at their centromeres. Reviews of the literature by White (*11*), Matthey (*12*), and Lima-de-Faria (*13*), emphasize the structural and evolutionary role of the centromere in chromosomal differentiation. Rothfels *et al.* (*5*) noted, in a subline of Earle's NCTC clone 929 of strain L cells, that the ruffled appearance of a telocentric "marker" chromosome persisted after a transformation to an isochromosome or equal-armed ruffled element. It is therefore a probable hypothesis that many of the telocentrics observed in the Chinese hamster cultures have arisen from either arm of a #3, 4, or 8 chromosome.

In addition to the V, P, C, and C-M/C cell strains, already described, a new cell strain X initiated in May, 1958, has been studied in the same way. The number of observations is, as yet, limited, but two aspects are of interest in the light of the findings in the other cell strains: 1) the X culture lost its classical chromosome pattern within the first 3 months of life *in vitro*, and 2) the commonest abnormality has been the addition of an extra medium-sized metacentric chromosome resembling a large #8. This has never been a feature of the other cell strains and, up to the present, telocentric chromosomes have not been observed in the X culture, a fact which would be in accord with the hypothesis outlined.

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PLATE 72

All figures are phase-contrast photographs of orcein-stained metaphase plates

FIGURE 1.—Classical diploid V metaphase from culture in the 10th month of life *in vitro*. × 2,500

FIGURE 2.—P-1 metaphase: 21 chromosomes; 1 normal #1, 1 aberrant #1 with deletion of half of 1 arm (*arrow 1*), 1 normal #2, 1 aberrant #2 with deletion of three fourths of 1 arm (*arrow 2*), 5 medium metacentrics (#3, 4, and 8), 6 small metacentrics (#5, 6, and 7), and 6 subterminals (#9, 10, and 11). × 2,500

FIGURE 3.—C-2 metaphase: 35 chromosomes; 2 normal #1, 1 aberrant #1 with deletion of half of 1 arm (*arrow 1*), 2 normal #2, 1 aberrant #2 with deletion of three fourths of 1 arm (*arrow 2*), 7 medium metacentrics (#3, 4, and 8), 10 small metacentrics (#5, 6, and 7), 9 subterminals (#9, 10, and 11), 2 telocentrics, and 1 large subterminal (*arrow LS*). *Insert:* Two large subterminal chromosomes from other similar C-2 metaphases showing, more clearly, the secondary constrictions. × 2,500

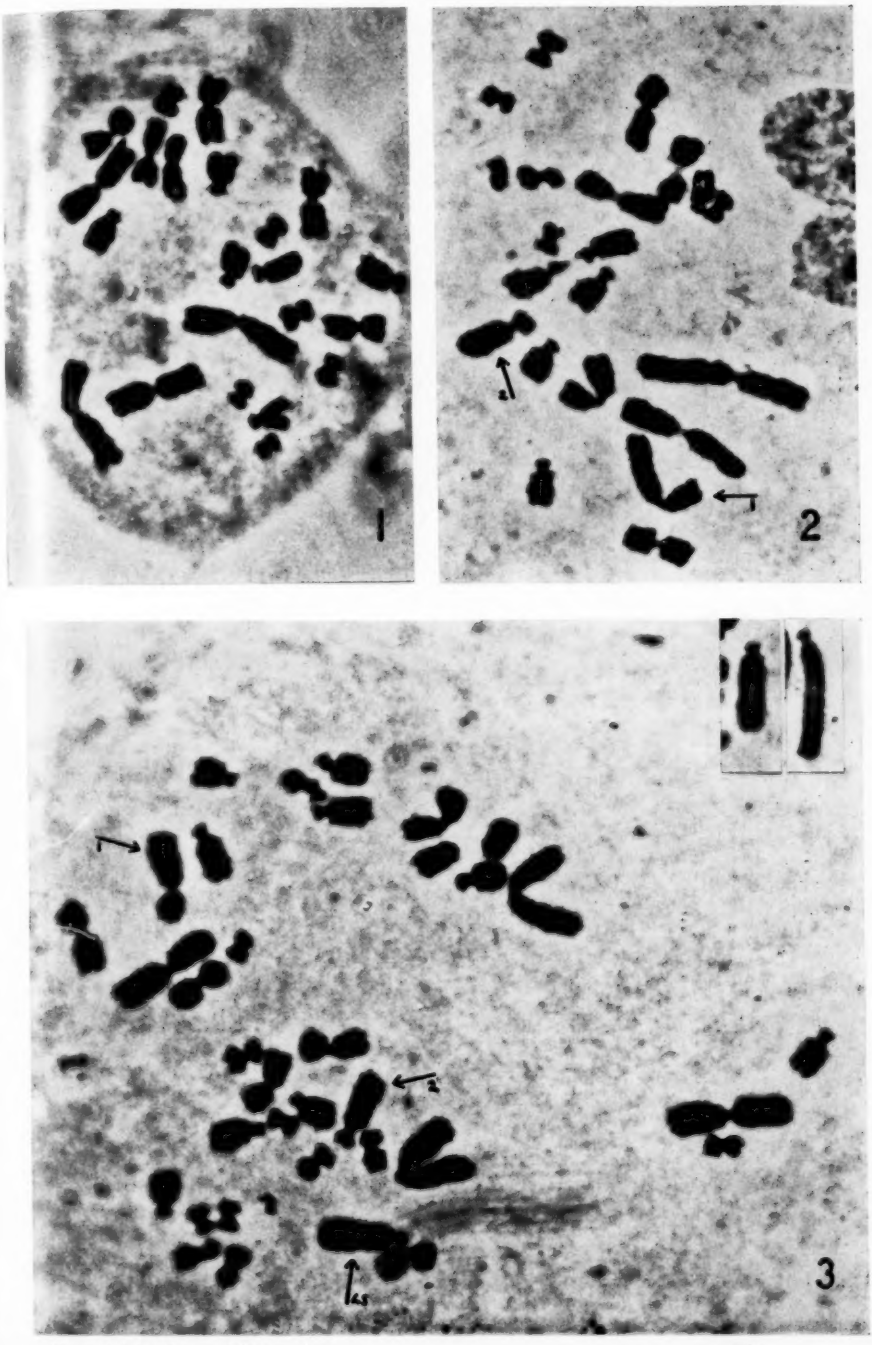

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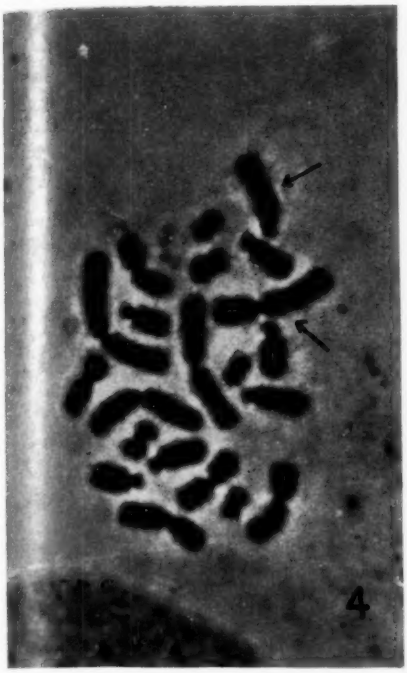
PLATE 73

FIGURE 4.—C-M/C metaphase: 21 chromosomes; 1 normal #1, 1 aberrant #1 with deletion of half of 1 arm (*arrow*), 2 normal #2, 5 medium metacentrics (#3, 4, and 8), 5 small metacentrics (#5, 6, and 7), 6 subterminals (#9, 10, and 11), and 1 large subterminal (*arrow*). × 2,500

FIGURE 5.—C-1 metaphase: 35 chromosomes; 2 normal #1, 1 aberrant #1 with deletion of half of 1 arm (*arrow*), 3 normal #2, 8 medium metacentrics (#3, 4, and 8), 10 small metacentrics (#5, 6, and 7), 9 subterminals (#9, 10, and 11), and 2 telocentrics (*arrows*). × 2,500

FIGURE 6.—C-1 metaphase: 36 chromosomes; 2 normal #1, 1 aberrant #1 with deletion of half of 1 arm (*arrow*), 3 normal #2, 9 medium metacentrics (#3, 4, and 8), 10 small metacentrics (#5, 6, and 7), 10 subterminals (#9, 10, and 11), and 1 telocentric (*arrow*). × 2,500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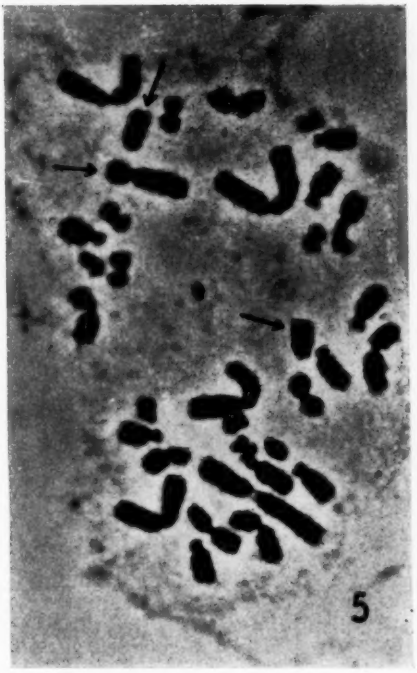
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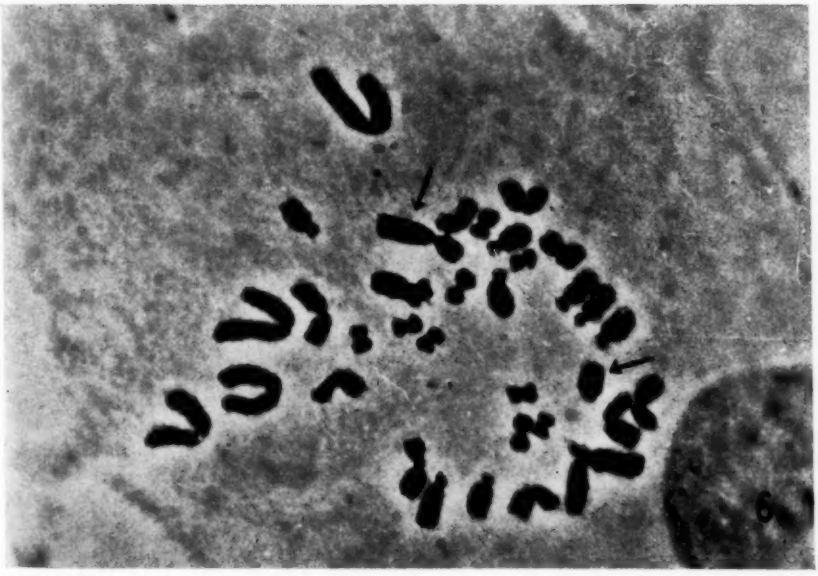
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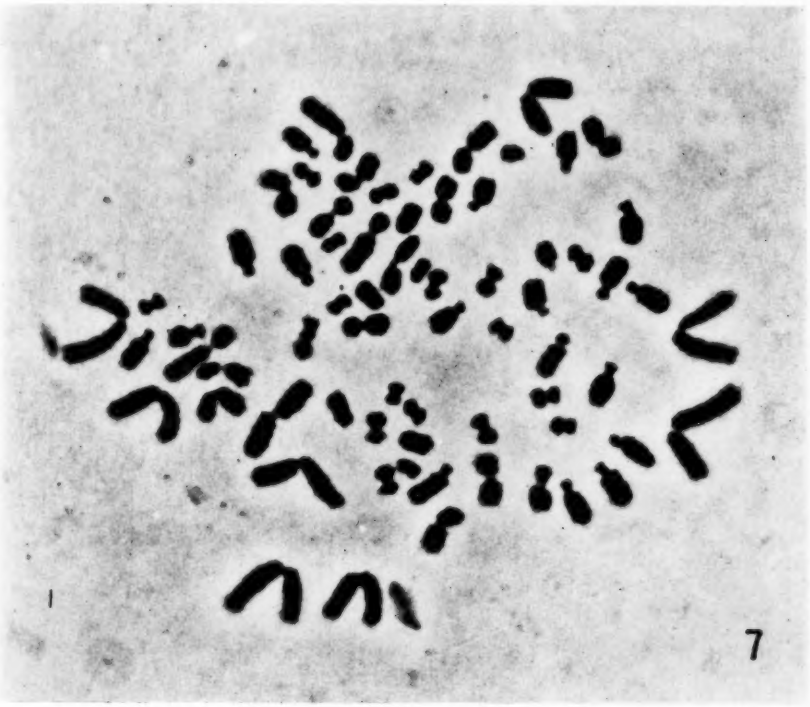
PLATE 74

FIGURE 7.—C metaphase: 69 chromosomes; 11 large metacentrics #1 and 2 (including 2 with deletions), 15 medium metacentrics (#3, 4, and 8), 20 small metacentrics (#5, 6, and 7), 18 subterminals (#9, 10, and 11), and 5 telocentrics. × 2,500

FIGURE 8.—C metaphase: 23 chromosomes; 2 #1, 2 #2, 1 #3, 2 #4, 2 #5, 4 #6 and 7, 2 #8, 2 #9, 4 #10 and 11, and 2 telocentrics (*arrows*). × 2,500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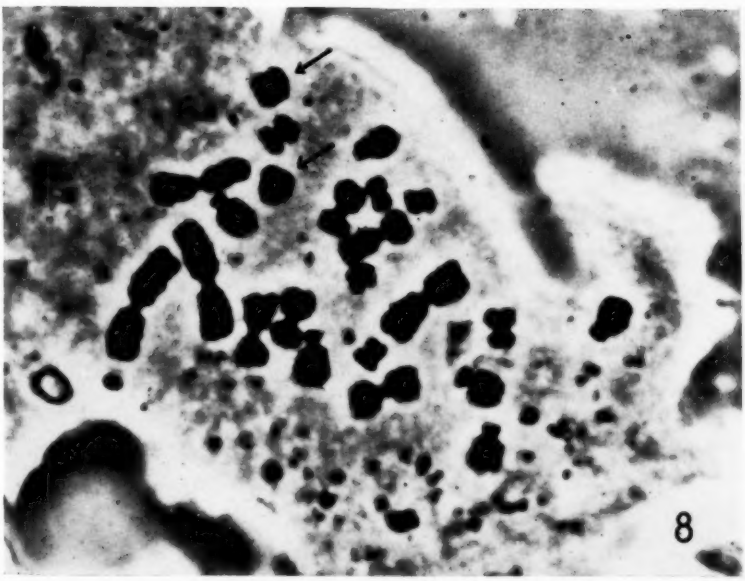
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PLATE 75

FIGURE 9.—C metaphase: 85 chromosomes. Marked polyploidy with aberrant chromosomes including rings and dicentrics (*arrows*). × 2,500

FIGURE 10.—C-M/C metaphase. Polyploidy with chromosome disintegration and the formation of a quadriradial. × 2,500

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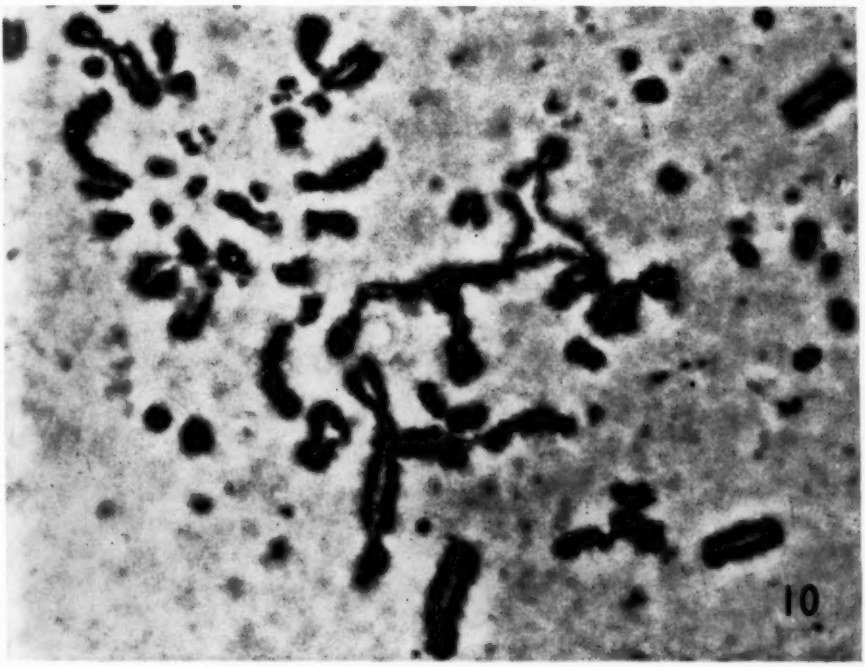
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Plate 76

Figure 11.—Polyploid C anaphase with a bridge and a fragment. × 1,500

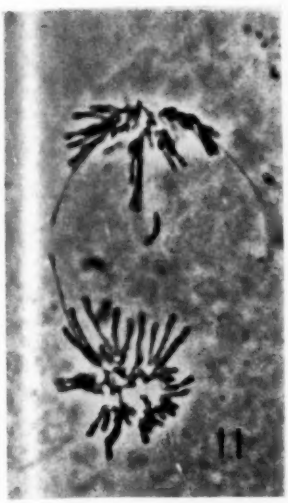
Figure 12.—Polyploid C telophase with 1 fragment. × 1,500

Figure 13.—Polyploid C-M/C anaphase with bridge having short arms and with fragment showing the point where sister reunion occurred. × 1,500

Figure 14.—Polyploid C-M/C anaphase with 1 crisscross bridge and 1 single bridge. × 1,500

Figure 15.—Polyploid C prophase showing stickiness and a number of fragments. × 1,500

Figure 16.—Polyploid C-M/C metaphase showing delayed orientation of the "short-armed" #1 chromosome and other chromosomes at the equatorial plate. × 1,500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16

Relationships Between Lymphocytic Choriomeningitis Virus, Amethopterin, and an Amethopterin-Resistant Lymphocytic Neoplasm in Mice[1]

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Summary

Amethopterin resistance was developed in the tetraploid lymphocytic neoplasm P288 by serial transfer of cells of the amethopterin-sensitive stem line through amethopterin-treated mice. The character of amethopterin resistance remained stable and heritable following the transfer of cells, for many transfer generations, through untreated mice. Lymphocytic choriomeningitis (LCM) virus did not influence this resistance since it was never found in the sensitive stem line or in the amethopterin-resistant sublines. However, the resistant subline (P288/AmR-II) was infected intentionally with the virus of LCM in a single exposure *in vitro* and carried it, as a passenger, through 39 successive transfers. Passage of this contaminated tumor through mice immune to LCM caused the virus to disappear, insofar as the tests were capable of detecting it, but virus did not disappear from amethopterin-treated immune mice bearing the tumor. It was also found that the resistant subline (free of LCM) of neoplasm P288 could grow progressively and kill certain mice of foreign strains when such mice were treated with amethopterin.—J. Nat. Cancer Inst. 22: 801–809, 1959.

The virus of lymphocytic choriomeningitis (LCM) given intracerebrally to mice ordinarily causes fatal meningitis, often accompanied by convulsions of a characteristic pattern. Extensive infiltration of the meninges, choroid plexus, and ventricles, by lymphocytes, has been repeatedly described as the characteristic microscopic lesion. The early reports of Armstrong *et al.*, Rivers and Scott, Traub, Finlay *et al.*, and other workers, describing lymphocytic choriomeningitis in humans and animals are documented by van Rooyen and Rhodes (*1*).

In certain instances large quantities of LCM virus in the brain do not cause death; for example, mice infected before birth (*1*), mice given total body X irradiation prior to virus injection (*2*), or mice made folic acid deficient by diet or amethopterin (*3*). Humoral antibodies against the

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

virus are difficult to demonstrate in mice, but solid immunity to challenge follows recovery from any form of the infection (*1*). Mice that escape death because of X-ray treatment or folic acid deficiency become virus carriers. Since X rays and folic acid deficiency suppress the lymphocytic reaction evoked in the meninges by LCM (*2*, *4*), it seems reasonable to assume that the immune response and the lymphocytic reaction are related.

The virus of LCM presents a problem to laboratories working with transmissible neoplasms of mice, because once the virus is acquired in some accidental fashion, by a tumor-bearing mouse, it may be carried along with subsequent transplants, probably indefinitely (*5*). Mice with tumors resistant to amethopterin are particularly vulnerable because of the tendency of the drug to promote the carrier state.

This report describes the development of an amethopterin-resistant lymphocytic neoplasm of the mouse, its intentional contamination with LCM virus, the effect of passage of the contaminated tumor through mice previously immunized against LCM, and the ability of amethopterin to promote its progressive growth in foreign strains of mice.

Materials and Methods

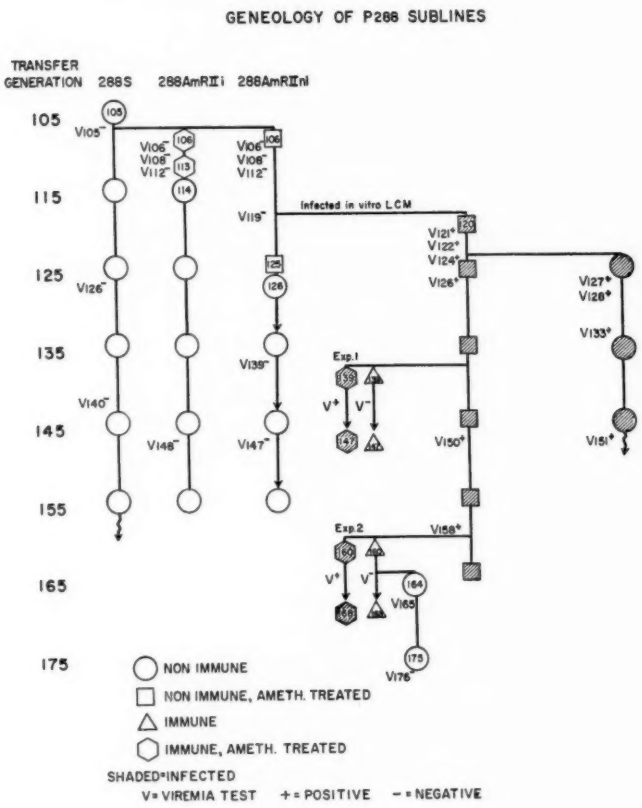
Noninbred Swiss mice free of LCM infection were used for virus detection. These and C57BL/6Jn and the (BALB/c × DBA/2)F_1 hybrids (referred to as CDF_1) were obtained from the Laboratory Aids Branch of the National Institutes of Health. C3Hf/Fg and Zf(C3Hf/Bi) were obtained from the colony of L. W. Law. Strains C57L/De, B10·LP/Sn, C57BL/10ScBs, C57BL/Ka and C57BL/10 *H-2*^{4} were supplied by D. E. Uphoff. Neoplasm P288 was used, which is a tetraploid lymphocytic neoplasm of DBA/2 origin previously described (*6*). Text-figure 1 gives the origin of the sublines.

Amethopterin (methotrexate) [4] 3 mg. per kg. body weight, was given subcutaneously in the interscapular region on alternate days, 3 times weekly. Toxicity was seldom seen because of the short duration of the experiments.

The LCM virus was from an intracerebrally (i.c.) passed strain (*7*). To contaminate the tumor, 0.2 ml. of ascites diluted with 1.8 ml. of saline was mixed with 1.0 ml. of 10^{-3} infected brain emulsion, allowed to stand 15 minutes at room temperature, and injected intraperitoneally (0.25 ml.). Mice were immunized by subcutaneous injection of 0.15 ml. of 10^{-3} brain emulsion, which was repeated after 2 weeks. Immunity was proved by i.c. challenge with homologous virus. Viremia was demonstrated by i.c. injection of heart blood (diluted 1/10) into 3 to 5 mice.

Mice used for virological studies were maintained on diets of whole-wheat bread soaked in powdered whole milk reconstituted with tap water, and were kept in glass jars. Those not infected with virus were kept in

[4] We are indebted to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for this drug.



TEXT-FIGURE 1.

plastic cages in another building, fed on Derwood pellets, and given tap water *ad libitum*.

Results

Development of Amethopterin-Resistant Tumor (P288/AmR-II)

The amethopterin-susceptible tumor, P288/S, was divided at the 105th transfer generation into 3 lines. One was carried thereafter in untreated mice, not immunized to LCM; one was carried in amethopterin-treated mice previously immunized against LCM; and one was transferred to amethopterin-treated, nonimmune mice (text-fig. 1). Tests for LCM made on heart blood of mice from the stem line, at the time of this division and later at the 126th and 140th passages, were negative; mice in this line have never shown clinical evidence of LCM infection. The subline passed through amethopterin-treated immune mice [P288/AmR-II(i)] was negative on viremia tests at the 106th, 108th, and 112th generations; after the 113th passage this tumor was carried in nonimmune untreated

TABLE 1.—*Effect of amethopterin on survival time of mice bearing uninfected sublines of P288*

Line	Transfer generation	Number of transfer generations		Cell dose	Number mice/ group	Survival (*days*)		Average percentage increase in survival time
		On amethopterin	Since last amethopterin			Control avg. (*range*)	Amethopterin-treated avg. (*range*)	
P288/S	122	—	—	10^5	8	12.1 (12–13)	34.2*(28–52)	182
	140	—	—	10^5	8	13.0 (9–16)	33.1†(29–39)	154
	167	—	—	2.7×10^5	6	9.8 (8–12)	27.8 (23–33)	183
P288/AmR-II(ni)	138	19	14	10^5	8	12.1 (11–14)	13.6 (10–17)	12.4
	149	19	25	10^5	6	13.0 (11–14)	14.3 (13–16)	10
	161	19	37	2.1×10^5	6	10.6 (10–13)	11.8 (8–14)	11.3
	163	19	39	5.3×10^5	6	9.6 (8–11)	10.5 (8–14)	9.3
	165	19	41	5.5×10^5	8	9 (7–10)	10.7 (10–12)	18.9
P288/AmR-II(i)	150	8	36	10^5	6	12.5 (11–14)	17.3 (16–18)	38.3

*Does not include 1 survivor in amethopterin-treated group.
†Does not include 2 mice that died of toxicity.

mice, and viremia tests were still negative at the 148th generation. The subline carried in amethopterin-treated nonimmune mice [P288/AmR-II (ni)] gave negative viremia tests at the 106th, 108th, 112th, and 119th generations; its 126th and subsequent passages were in untreated nonimmune mice, from which negative viremia tests were obtained at the 139th and 147th generations. It is evident that all these lines remained free of demonstrable infection with LCM. Both amethopterin-treated sublines became resistant to the drug, and this resistance continued after passage without amethopterin was resumed (table 1). The development of drug resistance in this instance appears clearly to have had no dependence upon LCM.

That LCM could accompany passage of this tumor was demonstrated by intentionally infecting the P288/AmR-II(ni) subline at the 119th generation (text-fig. 1), and subsequently carrying it in amethopterin-treated and untreated nonimmune mice. Virus was recovered from blood or ascites on repeated occasions, up to the 158th generation, 39 passages after contamination (*see* text-fig. 1).

Passage of LCM-Contaminated P288/AmR-II(ni) in Immune Mice

The amethopterin-resistant, LCM-contaminated tumor was implanted into LCM-immune mice in 3 separate experiments, with a total of 16 mice—8 of them treated with amethopterin, and 8 untreated. Viremia was demonstrated in 6 of the treated mice and in none of the untreated ones. Two of these experiments were extended by passing the tumor for 9 serial transfers through treated and untreated immune hosts. The results of these experiments are recorded in table 2.

In experiment 1, for the first 5 transfer generations, ascites instead of heart blood was inoculated intracerebrally into Swiss mice. This resulted

TABLE 2.—*Effect of amethopterin on recovery of LCM virus from tumor-bearing immune mice*

Passage generation	Virus recoveries/tests			
	Expt. 1*		Expt. 2	
	Treated	Untreated	Treated	Untreated†
1	1/2	0/2	2/2	0/2
2	1/2	0/2	—	—
3	1/2	0/2	1/1	0/1
4	1/2	0/2	2/2	0/2
5	1/2	0/2	2/2	0/2
6	1/1	0/1	2/2	0/2
7	—	—	2/2	0/2
8	1/1	0/2	2/2	0/2
9	1/1	0/2	2/2	0/2

*For the first 5 generations of experiment 1, tests for virus were made by intracerebral injection of ascites into general-purpose mice. All other tests were for viremia.

†In experiment 2, in addition to viremia tests, ascites was passed from untreated immune to nonimmune CDF_1 mice in the sixth, seventh, and eighth generations; these nonimmune mice were, in turn, tested for viremia, with negative results.

in the development of intracranial tumor growth, demonstrable between the 6th and 8th days. Heart blood from these mice was used to demonstrate virus by i.c. injection into fresh mice. This means that virus was given an opportunity to develop in susceptible mice before the definitive viremia test. It is a strong indication not only that the untreated tumor-bearing immune mouse became free of virus but that the tumor tissue itself had become free of virus. In the later transfers in experiment 1 (table 2), heart blood was used and no intracranial tumor growth appeared, but virus was still found in the heart blood of amethopterin-treated, immune mice after 9 transfer generations.

In experiment 2, heart blood was used throughout, and virus was consistently recovered from amethopterin-treated but not from untreated mice. In addition, in the sixth, seventh, and eighth transfer generations, ascites from untreated immune mice was transferred to nonimmune CDF_1 mice, which, in turn, were tested for viremia—all with negative results. Again it was found that viremia was directly demonstrable in treated, immune hosts throughout, and after 9 serial transfers.

In another experiment, the contaminated tumor was transferred through 5 generations of untreated, immune mice, beginning with the 159th passage. An undetermined number of cells was used to initiate the 159th and 160th passages, but inoculums of 100 cells each for the 161st to 163rd, inclusive, were used. Thereafter, ascites was carried in untreated, nonimmune mice by inoculums in excess of 10^5 cells (text-fig. 1). Viremia tests were negative at the 168th and 176th generations. Ascites at the latter passage was implanted subcutaneously into general-purpose mice, which were challenged intracerebrally with LCM 2 weeks later; they were not immune.

Transfer of the Uninfected Amethopterin-Resistant P288 Neoplasm to Foreign Strains

The histocompatibility-2 (*H-2*) locus largely governs the susceptibility and resistance to normal as well as to neoplastic tissue transplantation. A difference of phenotype at this locus, between a neoplastic cell population and a host, will prevent the progressive growth of nearly all transplanted neoplasms. Strain DBA/2 has the phenotype *H-2^d;* thus P288, which originated in this strain, will grow progressively in all DBA/2 mice and F_1 hybrids of DBA/2 and any other strain.

Fortuitously it was observed that P288/AMR-II(ni) appeared to grow progressively in amethopterin-treated noninbred Swiss mice (the *H-2* phenotype not determined). Accordingly, experiments were undertaken to determine whether this tumor would grow progressively and kill amethopterin-treated mice of foreign strains. In all, 8 different strains were observed; as might be expected, all CDF_1 mice died of the neoplasm whether treated with amethopterin or not (table 3).

When P288/AmR-II(ni) was inoculated into strain Zf mice (a subline of C3H which is *H-2^k*), 24 untreated controls showed transitory evidence of ascites development, while 38 of the 40 amethopterin-treated mice

TABLE 3.—*Effect of amethopterin on survival of various mouse strains inoculated with uninfected P288/AmR-II(ni) mice*

Strain	Sex	*H-2* type*	Cell dose	Transfer generation	Amethopterin (*3 mg./kg. body weight*)		Untreated		Amethopterin treated	
					Number treatments	Day begun	Number dead/number inoculated	Mean day of death	Number dead/number inoculated	Mean day of death (*range*)
CDF_1	M	$H\text{-}2^d/H\text{-}2^d$	10^3–10^5	138–165	5–8	1	34/34	10.8	34/34	12.2 (8–17)
Zf	M, F	$H\text{-}2^k$	1.7×10^5	159	7	1	0/6	—	6/6	11.0 (8–15)
Zf	M	$H\text{-}2^k$	2.1×10^5	161	5	1	0/8	—	8/8	9.5 (7–12)
Zf		$H\text{-}2^k$			4	4	—	—	8/8	8.2 (6–13)
Zf		$H\text{-}2^k$			11	6	—	—	6/8	12.3 (9–16)
Zf		$H\text{-}2^k$			7	1	0/10	—	10/10	14.6 (11–16)
Zf	F	$H\text{-}2^k$	5.5×10^5	165	9	1	0/6	—	0/5	—
Zf†	M	$H\text{-}2^k$	5.5×10^5	165	9	1	0/8	—	0/6	—
Zf†	M, F	$H\text{-}2^k$	5.5×10^5	165	9	1	0/9	—	7/9	16.2 (11–22)
C3Hf/Fg	F	$H\text{-}2^k$	5.5×10^5	165	2	1	0/11	—	4/11	8.0 (6–11)
C3Hf/Fg	M, F	$H\text{-}2^k$	9×10^5	175	6	1	—	—	9/10	10.6 (8–15)
C3Hf/Fg	M, F	$H\text{-}2^k$	9×10^5	175	6	1	0/6	—	6/6	12.6 (8–16)
B10·LP/Sn	M	$H\text{-}2^b$	5.3×10^5	163	7	2	0/6	—	5/7	15.2 (14–18)
B10·LP/Sn	M	$H\text{-}2^b$	9.2×10^5	175	7	1	0/6	—	0/5	—
C57L/De	M	$H\text{-}2^b$	5.3×10^5	163	9	1	0/24	—	0/24	—
C57BL/6Jn	M	$H\text{-}2^b$	5.3×10^5	163	7	1	0/10	—	0/11	—
C57BL/Ka	M	$H\text{-}2^b$	9.2×10^5	175	6	5	0/13	—	10/16	13.8 (10–16)
C57BL/10ScBs	M	$H\text{-}2^b$	5.3×10^5	163	6	1	1/10	—	11/11	12.1 (10–14)
C57BL/10 $H\text{-}2^d$	M	$H\text{-}2^d$	9.2×10^5	175						

*See references 8 and 9. Mice in these groups were untreated controls from previous experiments in which P288/AmR-II had been inoculated.
†Zf is the same as C3Hf/Bi.

developed progressive tumor growth and died. The 2 survivors appeared in a single group of 8 mice in which amethopterin treatment was delayed until the 6th post-transplantation day. When amethopterin treatment was begun on the 4th post-transplantation day, there was a more rapid development of ascites, and death occured. Untreated mice, which recovered from either P288/S or P288/AmR-II(ni), were reinjected by the resistant subline, and amethopterin failed to prevent the development of ascites and death.

The C3Hf/Fg subline, also *H-2*^{k}, responded similarly to Zf mice, as might be expected. In one experiment only 2 amethopterin treatments were given (days 1 and 3) and though the mice developed striking ascites, only 4 of the 11 inoculated died.

A number of *H-2*^{b} strains were used. In the two coisogenic lines (which differ in phenotype at another histocompatibility locus *H-3*), B10·LP/Sn (*H-2*^{b}) and C57BL/10ScBs (*H-2*^{b}), P288 grew progressively in amethopterin-treated mice (*8*). Other *H-2*^{b} strains, C57L/De, C57BL/6Jn, and C57BL/Ka rejected the neoplasm even though treated with amethopterin.

Strains C57BL/10ScBs and C57BL/10 *H-2*^{d} are coisogenic, with a single gene difference at the *H-2* locus. P288/AmR-II(ni) grew progressively in amethopterin-treated mice of both these strains. Untreated mice of strain C57BL/10 *H-2*^{d} developed a striking amount of ascites, which regressed in all but one case. These results are still preliminary as far as the larger aspect of histocompatibility relationships is concerned.

Discussion

The repeated recovery of LCM virus from amethopterin-treated immune mice inoculated with contaminated tumors, and the failure to demonstrate virus in untreated ones, is a further indication of a concept previously advanced (*10*) that this antimetabolite interferes with the immune response of the mouse to LCM. The effect of amethopterin on tumors in foreign strains of mice suggests an interference with the developing homograft reaction. Both these effects seem related in principle.

Previous reports have shown that in mice without tumors amethopterin did not suppress established immunity to LCM (*10*). This difference in the response of the tumor-bearing and non-tumor-bearing immune mouse to the drug suggests that it is the tumor cells which make possible the survival of virus in amethopterin-treated immune mice. Amethopterin appears to delay the lymphocytic reaction to LCM virus in i.c. inoculated, nonimmune mice (*4*), and it might be supposed that in the tumor-bearing immune mice under consideration here, the drug may foster virus survival by impeding a reaction between immune lymphocytes and tumor cells.

It could be conjectured that passage through amethopterin-resistant tumor cells altered the LCM virus so that the mouse, though immune to the parent virus strain, could not deal effectively with the altered one. But this argument can be dispelled by the observation that untreated immune mice rid themselves of demonstrable virus.

It is known that homograft reactions are preventable by separating the

graft from the host with a cell-excluding membrane (*11*). The present experiments on tumor growth in foreign strains are basically similar to homograft manipulations, and, since amethopterin prevents tumor destruction in these foreign hosts, it may be postulated that it acts like the cell-excluding membranes, *i.e.*, it inhibits access of innately resistant (immune) cells to the introduced tumor cells. Perhaps this is the way in which it favors LCM virus propagation in the immune tumor-bearing mouse. If this is so, it would indicate that in these mice the site of virus replication is the tumor-cell population. This point, since it has fundamental immunological significance, merits further study.

These experiments also show that neoplasm P288 was able to develop amethopterin resistance without the presence of LCM—a point that needs emphasis because of the sometimes accidental occurrence of this virus in mice used for passage of drug-resistant tumors.

Virus could conceivably persist in tumor cells in some latent form, not demonstrable by ordinary methods, after passage through immune mice. In passing the amethopterin-resistant contaminated tumor through immune mice, to exclude the virus, innoculums of 100 cells were used to reduce the likelihood of including variant cells, which might contain inapparent virus, possibly in some state resembling that found in lysogenic bacterial viruses.

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Prolongation of the Lifespan of Mice With Advanced Leukemia (L1210) by Treatment With Halogenated Derivatives of Amethopterin [1]

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Summary

Studies were conducted on the influence of 4 halogenated derivatives of amethopterin in increasing the lifespan of mice with systemic leukemia (L1210). Amethopterin was employed as a standard. 3′-Bromoamethopterin appeared to be moderately more effective than amethopterin, but not as effective as 3′-chloroamethopterin. The latter yielded increases in median survival time 13 to 34 percent greater than those observed with amethopterin. The dihalogenated derivatives 3′-bromo-5′-chloroamethopterin and 3′,5′-dichloroamethopterin were extremely effective in this system. Both drugs were capable of extending the median survival time of the mice for the full period of treatment (90 days) and beyond, even when treatment was initiated only several days before death of controls. Some of the animals were still alive after 6 months and were presumably cured. The fact that some of these mice did not accept reinoculation of the leukemia suggests that they may have become immune. The antileukemic action of the halogenated derivatives may be reversed by concomitant administration of citrovorum factor. The implications of the data are discussed with respect to therapy and underlying mechanism.—**J. Nat. Cancer Inst. 22: 811–823, 1959.**

A systematic program of investigation has been in progress in this laboratory on the influence of chemotherapeutic agents against systemic leukemia (L1210) in mice (*1–4*). An assay procedure was developed for this purpose in which treatment is withheld until the disease is widely disseminated and death is imminent (*1*). It was determined initially that the folic acid analogue amethopterin was consistently more effective in increasing the lifespan of mice with advanced leukemia (L1210) than were various known antileukemic agents (*4*). Subsequently, it was shown that several congeners of amethopterin, including adenopterin and N^{10}-ethylaminopterin, were as effective as amethopterin in this system (*5*). Halogenated derivatives of both aminopterin and amethopterin appeared to be of particular interest. 3′,5′-Dichloroaminopterin was more effective than aminopterin and 3′,5′-dichloroamethopterin more effective than

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

amethopterin in increasing the survival time of mice with advanced leukemia (*5*). This advantage in treatment with the 3′,5′-dichloroamethopterin was evident on 4 different schedules: twice daily, daily, every 2 days, and every 4 days. Control animals inoculated with this leukemia generally succumbed in 9 to 13 days. With the dichlorinated derivative, a median survival time of over 30 days was observed, and survival time beyond 50 days was not uncommon, even though treatment was delayed until 2 or 3 days before death. Regression of the local tumor at the site of leukemic inoculation was observed when doses were used that did not cause concomitant loss in body weight.

It was subsequently determined that the sample of 3′,5′-dichloroamethopterin contained 3′-chloroamethopterin as an impurity (*5*, *6*). Purified 3′,5′-dichloroamethopterin and the corresponding monochloroamethopterin were synthesized (*6*) and in preliminary tests both appeared to exert greater antileukemic action than amethopterin (*7*, *8*). The purified dichloroamethopterin appeared to be even more effective than the impure sample. Preliminary studies also indicated that the bromination of amethopterin increased antileukemic effectiveness (*7*, *8*).

In view of these results, a more detailed investigation was undertaken on the halogenated folic acid analogues. Because of the preliminary findings, it was considered possible that such studies might provide chemotherapeutic agents capable of eliciting extensive increases in survival time, and even possible cure of systemic leukemia (L1210). In the current report, data are presented on the antileukemic effectiveness of purified samples of 3′-chloroamethopterin, 3′,5′-dichloroamethopterin, 3′-bromoamethopterin, and 3′-bromo-5′-chloroamethopterin.[3] Comparisons are made with amethopterin as a standard.

Methods

The methods employed in this assay system with advanced leukemia have been described previously (*1–5*). The experiments were conducted with the lymphoid leukemia L1210, carried in DBA/2J male mice or (BALB/cAn × DBA/2J)F_1 hybrid male mice, transplanted to the hybrid male mice. The tumor-cell suspension was prepared from a mash of spleen cells and inoculated subcutaneously into the right hind leg (0.1 ml. of leukemic cell suspension per mouse). The inoculum concentration per mouse (500,000 to 1,000,000 cells) was 10 to 100 times greater than that required to give 100 percent deaths from tumor in a serial dilution of the inoculum in controls. Treatment was initiated when the local tumor at the site of leukemic inoculation was approximately 8 to 12 mm. in diameter. This was 7 to 9 days following leukemic inoculation, and 2 to 3 days prior to the median time of death of controls. The leukemia is systemic at this stage and is readily transmissible by transplantation of blood, spleen, or other organs (*4*). In the current experiments, trans-

[3] The compounds employed in this study were kindly provided by the Research Division, American Cyanamid Company, Pearl River, N.Y.: amethopterin (methotrexate, Lederle 7-5054; 7-8689); 3′-chloroamethopterin (Cl-25,330); 3′,5′-dichloroamethopterin (Cl-25,079), 3′-bromoamethopterin (Cl-J 25,970); 3′-bromo-5′-chloroamethopterin (Cl-B 27,783): citrovorum factor (Calco R-3631-148). For the synthesis of the halogenated derivatives *see* (*6*).

plantation of blood from randomly selected mice, at the time treatment was initiated, was invariably successful. Amethopterin and the halogenated derivatives of amethopterin were dissolved in 2 percent sodium bicarbonate. Citrovorum factor was dissolved in distilled water. The drugs were administered subcutaneously in the axillary region on a daily schedule, unless otherwise indicated, at 0.01 ml. per gm. of body weight of mouse. An attempt was made to administer the drugs in doses ranging from nontoxic to toxic levels. The animals were observed for mortality, body weight, and local tumor size.

In the experiment where a combination of citrovorum factor with 3′,5′-dichloroamethopterin or amethopterin was employed, treatment was administered daily from the 3rd through the 18th day following leukemic inoculation. The drugs were injected into opposite axillae, the citrovorum factor being given immediately before the antagonists.

Results

Table 1 presents summary results of 5 experiments in which the antileukemic activities of various halogenated derivatives of amethopterin are evaluated. Additional detailed data for 2 of the experiments are presented in text-figures 1 to 4. An experiment relative to the ability of citrovorum factor to reverse the antileukemic effect of 1 of the halogenated derivatives is summarized in text-figure 5.

For the various experiments, table 1 shows, for each compound, the median survival time obtained in the treatment of late leukemia at the dose most effective, as tested, in prolonging the lifetime of the mice. The calculated relative efficacy of drug treatment, shown in the last column of table 1, is obtained as the increase at optimal treatment level in median survival time over untreated controls divided by the corresponding increase for optimal amethopterin treatment.

3′-Chloroamethopterin was tested in 4 of the 5 experiments: J312, J320, J321, and J325. In these experiments, on daily treatment, it yielded increases in survival time ranging from 13 to 34 percent greater than those achieved with amethopterin. The optimal doses of 3′-chloroamethopterin ranged from 1.3 to 3.6 mg. per kg. daily, while for amethopterin the range was 0.47 to 1.3 mg. per kg. This comparison between the 2 drugs is brought out in text-figure 1 from experiment J321, which shows the median survival time obtained at each level of the compounds tested. Over about an eightfold range 3′-chloroamethopterin yielded a relatively stable median survival time which was greater than the best median survival obtained with amethopterin.[4]

In 2 experiments, J312 and J320, alternate day treatment was attempted. Here, too, 3′-chloroamethopterin was more effective than amethopterin, producing increases of 15 and 18 percent in survival time over amethopterin. On the altered schedule the optimal dose of 3′-chloroameth-

[4] With 3′-chloroamethopterin, equal median survival time was obtained with 0.48 and 2.16 mg./kg. treatment levels; the determination of the higher dose as being optimum (table 1, expt. J321) is based, in this instance, on the entire pattern of survival time rather than on the median only.

TABLE 1.—*Effectiveness of halogenated derivatives of amethopterin in treatment of advanced mouse leukemia L1210**

Experiment†	Compound	Dose range (*mg./kg.*)	Optimal dose level (*mg./kg.*)	Median survival time at optimal dose (*days*)	Median remaining lifetime—day of treatment equal 0 (*days*)	Relative median remaining lifetime—untreated controls equal 1	Increase in median survival time over controls (*days*)	Relative increase in median survival time—amethopterin increase equal 1
J312, daily from day 9	Amethopterin	0. 27–3. 5	0. 76	27	18	6. 0	15	1. 00
	3′-Chloroamethopterin	0. 45–45	1. 3	29	20	6. 7	17	1. 13
every 2 days from day 9	Amethopterin	2. 1–5. 8	3. 5	29	20	6. 7	17	1. 00
	3′-Chloroamethopterin	9. 7–45	9. 7‡	31. 5	22. 5	7. 5	19. 5	1. 15
	Controls	—	—	12	3	1. 0	—	—
J320, daily from day 8	Amethopterin	0. 28–3. 6	0. 47	25	17	5. 7	15	1. 00
	3′-Chloroamethopterin	0. 28–10	2. 2	29	21	7. 0	18	1. 20
	3′,5′-Dichloroamethopterin	0. 47–28	28‡	32. 5	24. 5	8. 2	21. 5	1. 43
every 2 days from day 8	Amethopterin	0. 78–17	3. 6	28	20	6. 7	17	1. 00
	3′-Chloroamethopterin	1. 3–46	10	31	23	7. 7	20	1. 18
	3′,5′-Dichloroamethopterin	2. 2–129	129‡	29. 5	21. 5	7. 5	18. 5	1. 09
	Controls	—	—	11	3	1. 0	—	—
J321, daily from day 7	Amethopterin	0. 28–3. 6	1. 3	24. 5	17. 5	5. 8	14. 5	1. 00
	3′-Chloroamethopterin	0. 17–10	2. 2	28	21	7. 0	18	1. 24
	3′,5′-Dichloroamethopterin	2. 2–129	77	35	28	9. 3	25	1. 72
	3′-Bromoamethopterin	0. 17–129	2. 2	25	18	6. 0	15	1. 03
	Controls	—	—	10	3	1. 0	—	—
J325, daily from day 8	Amethopterin	0. 28–3. 6	1. 3	29	21	10. 5	19	1. 00
	3′-Chloroamethopterin	0. 17–6. 0	3. 6	35. 5	27. 5	14	25. 5	1. 34
	3′-Bromoamethopterin	0. 28–10	3. 6	31	23	12	21	1. 11
	3′-Bromo-5′-chloroamethopterin	0. 47–129	129‡	74. 5	66. 5	33	65. 5	3. 4
	Controls	—	—	10	2	1. 0	—	—

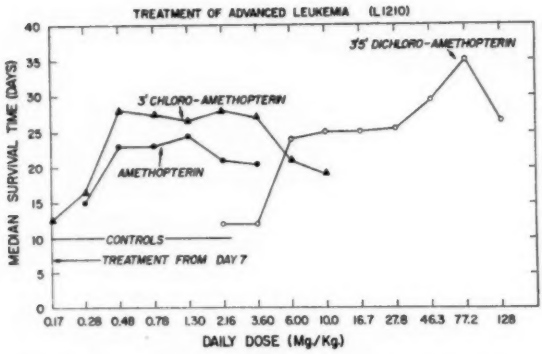
J330, daily from day 7	Amethopterin	0.48–2.3	0.60	29	22	8.8	19.5	1.00
	3′,5′-Dichloroamethopterin	14–200	82	>100	>93	>37	>90.5	>4.6
	3′-Bromo-5′-chloroamethopterin	14–200	102	>100	>93	>37	>90.5	>4.6
	Controls	—	—	9.5	2.5	1.0	—	—

*Ten mice at each dose level for each compound; 10 mice in each control group.

†Z line of tumor carried in DBA/2J male stock used in experiments J312, J321, and J330.

BB line of tumor carried in hybrid male mice (BALB/cAn × DBA/2J)F_1 used in experiments J320 and J325.

‡Optimal level not clearly established since best result observed occurs at an extreme of the dose range employed.


TREATMENT OF ADVANCED LEUKEMIA (L1210)
MEDIAN SURVIVAL TIME (DAYS)
DAILY DOSE (Mg/Kg)
3',5' DICHLORO-AMETHOPTERIN
3' CHLORO-AMETHOPTERIN
AMETHOPTERIN
CONTROLS
TREATMENT FROM DAY 7
0.17 0.28 0.48 0.78 1.30 2.16 3.60 6.00 10.0 16.7 27.8 46.3 77.2 128

TEXT-FIGURE 1.—Efficacy of amethopterin and 2 of its chlorinated derivatives in the treatment of advanced mouse leukemia (L1210). Data shown are from experiment J321, but do not include results for 3′-bromoamethopterin. The agents are tested over a series of dose levels to determine the maximal effectiveness of each in increasing the survival time of the tumor-bearing host. There were 10 mice at each dose level for each agent. This experiment is summarized in table 1.

opterin was, as before, higher than for amethopterin, *ca.* 10 mg. per kg. for 3′-chloroamethopterin and 3.5 mg. per kg. for amethopterin. In these experiments the drugs were somewhat more effective on an alternate day schedule than on a daily schedule.

3′-Bromoamethopterin was tested in 2 experiments, J321 and J325. In each experiment, the optimal dose was the same as for 3′-chloroamethopterin—2.2 and 3.6 mg. per kg. daily. 3′-Bromoamethopterin appeared to be only moderately more effective than amethopterin in these experiments. In the 2 experiments the increases in survival time were 3 and 11 percent beyond those for amethopterin.

3′,5′-Dichloroamethopterin was tested in experiments J320, J321, J330, and 3′-bromo-5′-chloroamethopterin in experiments J325 and J330. The optimal doses of these compounds proved so much greater than those of amethopterin that in experiment J320 the use of insufficiently high dose levels resulted in failure to uncover fully the high antileukemic efficacy of the dichlorinated agent.

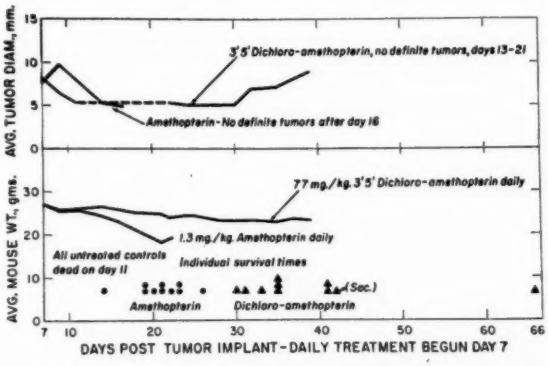
The 3′,5′-dichloroamethopterin was tested at sufficiently high dose levels in experiments J321 and J330. In J321, at an optimal dose of 77 mg. per kg. daily (*cf.* text-fig. 1), it yielded a 72 percent increase in antileukemic efficacy over amethopterin. On retest in J330, discussed in greater detail, the drug showed even greater efficacy.

Text-figure 2 compares the progressive changes occurring for host and local tumor at the site of leukemic inoculation in animals optimally treated with amethopterin or 3′,5′-dichloroamethopterin in experiment J321. Optimal treatment with the dichlorinated compound occasioned only gradual weight loss for the host, but caused the rapid disappearance of palpable tumors at the site of leukemic inoculation. Local tumors subsequently reappeared among these mice. With amethopterin treat-

ment, mice showed somewhat more rapid weight loss, and it took longer for the tumors to regress. Tumors did not reappear among these mice, but this may have been because they succumbed relatively early to drug toxicity.

In experiment J325 the highest dose level of 3′-bromo-5′chloroamethopterin employed, 129 mg. per kg. daily, was 3.4 times as effective as the optimal dose of amethopterin, 1.3 mg. per kg., in increasing the survival time of the leukemic mice. Even though treatment was initiated only 2 days before the median death time of untreated mice, there was a total of 17 survivors on day 90, the last treatment day, among the various groups treated with the agent.

The history of these long-term survivors is of interest. Between days 91 and 200, 12 mice became moribund and were killed, or died, some with



TEXT-FIGURE 2.—Effect of optimal daily treatment with amethopterin or 3′,5′-dichloroamethopterin on tumor growth, animal weight, and survival time in the chemotherapy of advanced mouse leukemia (L1210). Data are from experiment J321 (*cf.* table 1, text-fig. 1) and are for the drug levels in that experiment which yielded the most extensive survival time. Average animal weights and tumor diameters are shown only when based on 3 or more survivors.

and some without external evidence of tumor.[5] Seven mice were killed while moribund, between days 91 and 113. Subcutaneous transplantation of spleen tissue from these mice led in every instance to development of tumors among recipient mice. Three of the mice that were killed had developed tumors in the maxillary area, and tissue from these tumors was also transplanted successfully. One mouse was killed on day 200. Transplantation of its spleen, too, was successful.

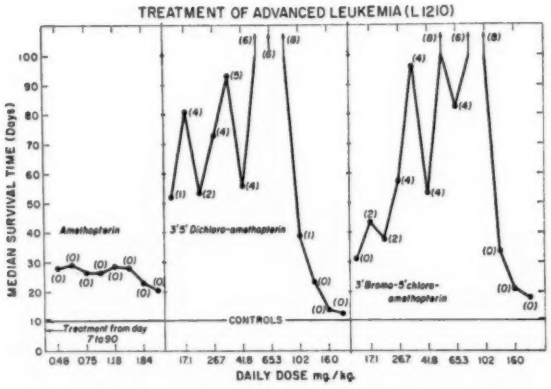
On day 204, some 16 weeks after treatment was discontinued, there were still five 3′-bromo-5′-chloroamethopterin-treated mice alive and apparently in good condition. These were reinoculated with heavy concentrations of leukemic cells (approximately 10^6 cells per mouse). Among

[5] None of these mice showed evidence of tumor at the original site of leukemic inoculation. Five showed tumor growth in the maxillary area. One had an apparent tumor outgrowth from one of the eye sockets. Several had internal tumors. A study of tumors from leukemic mice that survive for an extended period, as a result of therapy, will be reported separately.

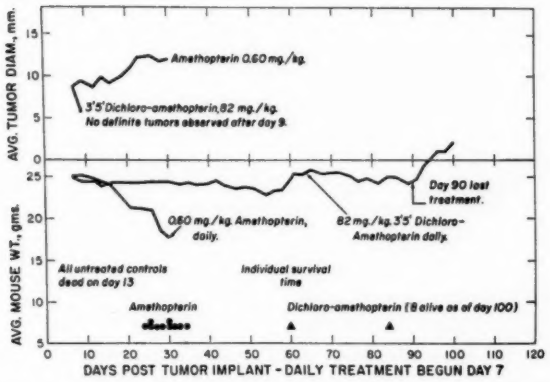
these 5 reinoculated mice there occurred 1 early death from tumor (day 8 following leukemic reinoculation) and 1 later death from tumor (day 29). The reinoculation of leukemic cells failed in the remaining 3 mice. They showed no evidence of leukemic growth and appeared in healthy condition at 30 days post reinoculation. Ten non-tumor-bearing controls in the experiment and 2 mice, in which earlier inoculation at low concentrations (1/1000 of experimental inoculum) had proved unsuccessful in the tumor titration series, were also reinoculated on day 204. These animals all succumbed to leukemic growth in 8 to 11 days.

The two dihalogenated compounds were tested simultaneously in experiment J330, and appeared to be of approximately equal therapeutic effectiveness. Both compounds proved so effective against the tumor (text-fig. 3) that at several dose levels median survival time could not be computed as of day 100. With the attainment of such extensive survival time, percentage survival rather than median survival time may be a more appropriate endpoint for measuring drug efficacy. On day 100 there remained a total of 41 survivors out of the 130 mice treated at the various levels of 3′,5′-dichloroamethopterin, with 8 survivors among 10 animals at the treatment level of 82 mg. per kg. daily. With 3′-bromo-5′-chloroamethopterin, 42 out of 130 mice survived to day 100, 8 of 10 mice surviving at the 102 mg. per kg. daily treatment level.

Text-figure 4 (expt. J330) shows that mice receiving the optimal treatment level of the dichlorinated compound maintained their weight during the course of treatment. When treatment was discontinued on day 90 there was actually a rapid rise in body weight. As in the previous experiment (J321; *cf.* text-fig. 2) the local tumors disappeared early at the site of leukemic inoculation. In contrast to the previous experiment,


TREATMENT OF ADVANCED LEUKEMIA (L1210)
MEDIAN SURVIVAL TIME (Days)
Amethopterin
3'5' Dichloro-amethopterin
3'Bromo-5'chloro-amethopterin
CONTROLS
Treatment from day 7 to 90
0.48 0.75 1.18 1.84 1.71 2.67 4.18 6.53 1.02 1.60 1.71 2.67 4.18 6.53 1.02 1.60
DAILY DOSE mg./kg.

TEXT-FIGURE 3.—Efficacy of amethopterin and 2 of its dihalogenated derivatives in the treatment of advanced mouse leukemia (L1210). Data are from experiment J330 (*cf.* table 1). The median survival time obtained employing 10 mice is shown at each level of daily treatment for each agent. Median survival time in excess of 100 days is indicated by arrows pointing upward. Figures in parentheses are the number of 100-day survivors in the various experimental groups.


AVG. TUMOR DIAM., mm.
Amethopterin 0.60 mg./kg.
3'5'Dichloro-amethopterin,82 mg./kg.
No definite tumors observed after day 9.
Day 90 last treatment.
0.60 mg./kg. Amethopterin, daily.
82 mg./kg. 3'5' Dichloro-Amethopterin daily.
AVG. MOUSE WT., gms.
All untreated controls dead on day 13
Individual survival time
Amethopterin
Dichloro-amethopterin (8 alive as of day 100)
DAYS POST TUMOR IMPLANT - DAILY TREATMENT BEGUN DAY 7

TEXT-FIGURE 4.—Effect of optimal daily treatment with amethopterin or 3′,5′-dichloroamethopterin on tumor growth, animal weight, and survival time in the chemotherapy of advanced mouse leukemia (L1210). Data are from experiment J330 (*cf.* table 1, text-fig. 3) and are for the drug levels in that experiment which yielded the most extensive survival times. Average animal weights and tumor diameters are shown only when based on 3 or more survivors.

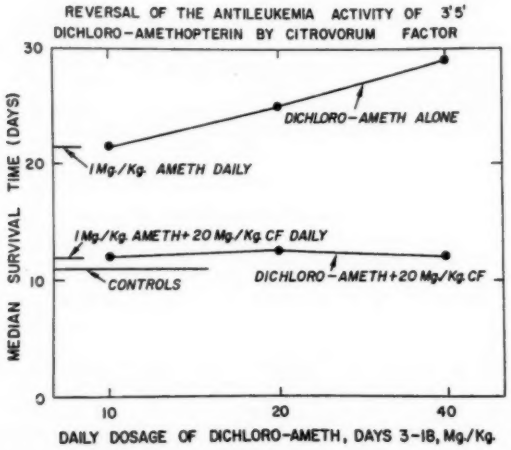
In this experiment, treatment at higher amethopterin dosages resulted in many tumors not being palpable for varying periods. When mice did not succumb early to drug toxicity, the tumors generally reappeared. At all levels of 3′,5′-dichloroamethopterin tested (14–200 mg./kg. daily) all tumors disappeared early. In a few instances, generally only at the lower dose levels, tumors subsequently reappeared. At the lower treatment levels, mice generally gained weight during the course of treatment.

As of day 130, a third death, on day 123, had occurred in the optimally treated 3′,5′-dichloroamethopterin group. Remaining mice were all apparently tumor free and ranged from 29 to 32 gm. in weight.

however, the local tumors did not reappear. Tumors disappeared even at the lower treatment levels and it was only at such lower levels that occasional tumors subsequently reappeared. The optimal dosage of amethopterin in this experiment was lower than usual, 0.60 mg. per kg. daily, and at this treatment level local tumors increased in size while average mouse weight decreased. At higher treatment levels tumors did disappear, subsequently reappearing when mice did not succumb too soon to drug toxicity. Since substantially the same pattern of results occurred in this experiment for both of the dihalogenated compounds, results for only one are shown graphically in text-figure 4.

The experiment relative to the reversibility of the antileukemic effect of 3′,5′-dichloroamethopterin by concomitantly administered citrovorum factor is reported in text-figure 5. As can be seen, citrovorum factor reversed the therapeutic effect of both amethopterin and its halogenated derivative. Dichloroamethopterin at 3 dose levels and amethopterin at 1 dose level significantly increased the survival time of the leukemic mice. Simultaneous administration of citrovorum factor prevented the increases in survival time. In this experiment treatment had been initiated prior

to the appearance of the local tumor at the site of leukemic inoculation. The dichloroamethopterin and the amethopterin retarded the growth of the local tumors. This, too, was overcome by concomitant administration of citrovorum factor. The local tumors in the groups treated with dichloroamethopterin or amethopterin plus citrovorum factor grew at approximately the same rate as untreated controls.



TEXT-FIGURE 5.—Reversal of the antileukemic efficacy of daily amethopterin or 3′,5′-dichloroamethopterin by the concomitant administration of citrovorum factor. The increased median survival time observed in daily treatment with either of the antifolics (1 mg./kg. daily of amethopterin; 10, 20, or 40 mg./kg. daily of 3′,5′-dichloroamethopterin) was nullified by concomitant treatment with citrovorum factor. In this experiment (J322, not covered in table 1), treatment of the mouse leukemia (L1210) was initiated relatively early, day 3, and was continued for 16 days. Ten mice per dose level.

Discussion

The results of the current experiments have a wide range of implications.

Primarily the study provides an experimental demonstration indicating the feasibility of controlling systemic leukemia by means of chemotherapy. With 3′,5′-dichloroamethopterin and 3′-bromo-5′-chloroamethopterin, it was possible to check the leukemic process in mice for an extensive period, even when treatment was started after the disease had infiltrated the various organs of the body. If left untreated, the animals succumbed in 2 to 3 days. When the mice were treated with well-tolerated doses of the dihalogenated derivatives of amethopterin, the local tumors at the site of leukemic inoculation generally regressed and the mice showed a uniformly extensive increase in lifespan.[6] Many of the mice

[6] The factors responsible for the dichlorinated derivative producing longer survival time in experiment J330 than in J321 are not clear. The same subline of leukemic cells was employed in both experiments and the optimal dose levels were in the same range. However, it should be noted that amethopterin also gave more extensive survival time in J330 than in J321. It is indicated that greater absolute variation in individual survival time may result with the more effective drugs.

lived the full period of therapy (90 days post inoculation of leukemia) and longer. A portion of the mice succumbed following withdrawal of therapy, but some of the animals were alive and healthy at 6 months following inoculation with leukemia. Whether mice such as the latter are cured of the disease or whether they still harbor leukemic cells has as yet not been determined. Frequently, when tail blood was taken from healthy-appearing long-term survivors and implanted into untreated mice, the implant failed to take (*9*). However, in some of these cases, the donor animal subsequently showed evidence of disease, and at that time transfer of blood did result in tumor growth. In any event, whether or not specific long-term survivors are indeed cured of the disease, it should be pointed out that since the normal lifespan of a mouse is approximately 2 years, the 6-month survival represents a very substantial increase in lifespan. This is even more noteworthy when it is considered that these mice would ordinarily have died of leukemia in just a few days.

In previous studies in this laboratory (*1–5*), such extensive survival time of mice with advanced leukemia L1210 had not been observed. For mice with advanced leukemia, the median survival time with amethopterin, formerly the most effective drug available in this system, is characteristically from 20 to 30 days, with an occasional animal surviving up to 40 days, but rarely beyond 50 days. With amethopterin, the demonstration of survival time up to 90 days or beyond, for this leukemia, has been limited to instances in which the treatment is initiated early in the course of the disease (*10–13*) when the disease is relatively localized.

The current results emphasize the importance of quantitative structure-activity studies in groups of chemotherapeutically active agents. What are the relationships of structural alterations to increased antitumor specificity? How does halogenation of amethopterin increase therapeutic activity in this system? The reversibility of antileukemic effect of dichloroamethopterin and of amethopterin by citrovorum factor is suggestive of similar underlying mechanism of action. Further similarity was observed in the ability of mono- and dichlorinated derivatives of amethopterin, as well as of amethopterin, at doses effective in increasing survival time of mice with systemic leukemia, to diminish formate incorporation in protein and nucleic acid adenine of leukemic-infiltrated spleen (*8*). On a molar basis the antitumor activity of the monohalogenated derivatives was not diminished, but was severely reduced for the dihalogenated derivatives. However, host tolerance was markedly increased with halogenation. The increased optimal dose of approximately threefold for the monohalogenated derivatives, and of the order of 100-fold for the dihalogenated derivatives of amethopterin, resulted in the increased therapeutic effect. A similar effect of halogenation has also been noted for derivatives of aminopterin (*5*, *7*).

It is indicated in the present experiments that, with extensive treatment, altered forms of the leukemia are being encountered. During treatment, local tumors could reappear after periods of regression; in other instances mice at tolerated treatment levels succumbed to diffuse rather

than palpable leukemias. Some of the tumors, recovered late in the course of the disease, were slow growing, whereas others appeared to retain the original rate of growth. Recovered tumors showed varying degrees of sensitivity and cross-resistance to amethopterin (*9*). These altered forms may resemble the "late recurrences" which were found in the plasma-cell neoplasm 70429 treated with glutamine antagonists (*14*). The ability to extend the lifespan of mice with systemic leukemia for such an extensive period provides material for the study of the emergence of resistance as well as the properties of the resistant forms. If drugs are found effective specifically for the resistant forms, their use in conjunction with presently effective agents could be warranted.

The current observation that long-term survivors of advanced leukemia may not accept reimplantation of the leukemia suggests that the surviving animals may have developed immunity. While the possibility exists that administration of the drug may have created an unsuitable environment for successful reinoculation, this seems unlikely since treatment was discontinued 114 days previously. If this phenomenon is indeed an immunological response, it will be of interest to determine whether it is somehow related to the hybrid environment or whether it can occur in the parent DBA/2J strain. Interestingly, the parent strains DBA/2J and BALB/cAn have corresponding histocompatibility genes at the *H-2* locus, (*15*).

Since the demonstration by Farber (*16*) that the folic acid antagonist aminopterin may prolong the lifespan of children with acute leukemia, there has been continued interest in the use of folic acid antagonists in clinical studies. 3′, 5′-Dichloroaminopterin is included among the various folic acid antagonists used by Farber (*17*). The chlorinated derivatives of amethopterin are currently under clinical investigation (*18*). To what extent the halogenated derivatives of amethopterin may provide additional useful tools for clinical study is difficult to predict. Regardless of clinical outcome, it should be emphasized that even though the current studies are conducted in mice they lend support to the concept that successful chemotheraphy of human leukemia is indeed possible.

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The Biosynthetic Preparation of C^{14}-Labeled *N*-(1- and 3-Hydroxy-2-fluorenyl) acetamides, and Their Metabolism in Rats and Guinea Pigs [1,2,3]

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Summary

The C^{14}-labeled *ortho*-hydroxylated metabolites of the carcinogen *N*-2-fluorenylacetamide, *N*-(1- and 3-hydroxy-2-fluoren-9-C^{14}-yl)acetamides, were prepared by biosynthesis in the rat. The metabolism of these compounds was studied in rats and guinea pigs after intraperitoneal injection. A larger percentage of a dose of either compound was excreted in the urine than in the feces. In rats and guinea pigs, 60 to 75 percent, and 90 percent, respectively, of the urinary radioactivity was in the form of glucuronides. The injected 1-hydroxy compound was excreted in the urine by rats as the 1-hydroxy, the 1,5-dihydroxy, and the 1,7-dihydroxy derivative, whereas guinea pigs eliminated mostly the 1,7-dihydroxy compound. About 69 percent of the urinary radioactivity was found to be the 3-hydroxy derivative: 8 percent as the 3,5- and 9 percent as the 3,7-dihydroxy derivative after administration of the 3-hydroxy compound to rats. Guinea-pig urine included 47 percent of the 3-hydroxy and 38 percent of the 3,7-dihydroxy derivative. Radioactivity from both injected compounds was present in all rat tissues examined. More isotope was tightly bound to the proteins of rat liver after injection of the 1-hydroxy derivative than after *N*-2-fluorenylacetamide, whereas the 3-hydroxy isomer gave lower levels. The specific activity of guinea-pig liver proteins was lowest after *N*-2-fluorenylacetamide, and somewhat higher after the 1- and 3-hydroxy compound. These results are discussed in relation to the species differences found between rats and guinea pigs, especially in regard to the apparent resistance of the latter species to carcinogenesis by *N*-2-fluorenylacetamide.—**J. Nat. Cancer Inst. 22: 825–844, 1959.**

A number of studies in various laboratories have shown that *N*-2-fluorenylacetamide (2-FAA), a potent carcinogen in the rat, appears to be

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[2] Presented in part at the 49th annual meeting of the American Association for Cancer Research, April 11-13, 1958, Philadelphia, Penna. Proc. Am. Assoc. Cancer Res. 2: 355, 1958.

[3] The *Chemical Abstracts'* system of nomenclature is used in this paper. Alternate names are given here for the reader's convenience: *N-(1-hydroxy-2-fluorenyl)acetamide*, 1-OH-2-FAA, 1-hydroxy-2-acetylaminofluorene, 2-acetylamino-1-hydroxyfluorene, 2-acetamido-1-fluorenol; *N-(3-hydroxy-2-fluorenyl)acetamide*, 3-OH-2-FAA, 2-acetylamino-3-hydroxyfluorene, 2-acetamido-3-fluorenol; *N-2-fluorenylacetamide*, 2-FAA, 2-acetylaminofluorene, 2-acetamidofluorene; *N-(7-hydroxy-2-fluorenyl)acetamide*, 7-hydroxy-2-acetylaminofluorene.

[4] With the able technical assistance of Mrs. A. Parker.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

noncarcinogenic for the guinea pig (*cf. 1*). This difference in biological results in the 2 species led to comparative metabolism experiments with the chemical for an explanation of this behavior. Miller and Miller (*2*) observed a good correlation between the carcinogenic effect of 2-FAA in several species and the level of protein-bound radioactivity in the livers of animals fed the C^{14}-labeled compound. Thus, in contrast to rat liver proteins, guinea-pig liver proteins exhibited only slight isotopic enrichment, a result also obtained in this laboratory (*1, 3*). A further important difference between the 2 species was in the composition of the urinary metabolites of 2-FAA (*1, 4*). Whereas rats produced moderate amounts of the *ortho*-hydroxylated derivatives of 2-FAA, *i.e.*, *N*-(1-hydroxy-2-fluorenyl)acetamide and *N*-(3-hydroxy-2-fluorenyl)acetamide, guinea-pig urine contained negligible amounts of these compounds.

This fact pointed toward a constitutional dissimilarity between the 2 species, which might have a bearing on the lack of carcinogenic effect of 2-FAA in the guinea pig. Hence, it was of considerable interest to devise an experiment which might reveal whether the lack of protein binding after the administration of 2-FAA was due to the failure of guinea pigs to produce the *ortho*-hydroxylated derivatives, or whether guinea-pig livers did not, perhaps, possess the kind of proteins required for binding the metabolites of 2-FAA. To this end, these *ortho*-hydroxylated derivatives of 2-FAA, labeled with C^{14}, were prepared, and the activity bound to the liver proteins after their administration to guinea pigs was determined. In addition, some of the urinary metabolites were identified and estimated. For comparative purposes, parallel studies on the distribution of radioactivity in the tissues, protein binding in the liver, and the urinary metabolites were performed in rats.

Materials and Methods

Isolation of N-(*1-hydroxy-2-fluoren-9-*C^{14}*-yl)acetamide and of* N-(*3-hydroxy-2-fluoren-9-*C^{14}*-yl)acetamide.*—A fairly stable suspension of 35.4 mg. or 1.06×10^9 counts per minute (cpm) of 2-FAA of high specific activity (6.6 mc/mmole) (*5*) in 4.5 ml. of an acacia solution [6] (*6*) was made by means of a motor-driven, all-glass homogenizer. Equal amounts of this suspension were injected intraperitoneally into 4 female Buffalo strain rats, weighing about 160 gm., which were transferred to glass metabolism cages (2 rats per cage). After 48 hours a second dose of a total of 31.7 mg. of labeled 2-FAA (9.51×10^8 cpm) was injected, divided equally among the 4 animals. Forty-eight hours later, the animals were autopsied and the organs removed for use in another study.

The pooled urine sample for the first 48-hour period contained 514 million cpm and in the second 48-hour period, 461 million cpm. The feces in the same time intervals accounted for 97 and 74 million, or a total of 171 million cpm.

The combined urines (253 ml., 975 million cpm) were buffered to *p*H 6 by means of 10 ml. of 1 M acetate buffer and extracted 5 times with 200-

[6] Seven gm. of acacia and 0.9 gm. of sodium chloride were made to 100 ml., boiled briefly, and filtered.

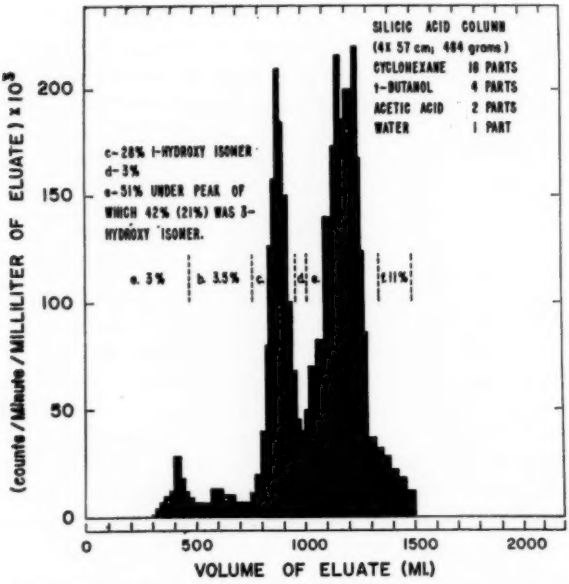
ml. portions of freshly distilled ether. The ether extract contained 105 million cpm (representing the amount of unconjugated metabolites) and the aqueous phase 800 million cpm. The latter was incubated for 40 hours with 250 mg. of bacterial β-glucuronidase and an equal amount of Taka-Diastase. An ether extraction (5 × 200 ml.) then removed 467 million cpm and left 297 million cpm in the aqueous layer. This ether extract, comprising the metabolites conjugated with glucuronic and sulfuric acid, was taken to dryness and the residue dissolved in 3 ml. of *tert.*-butanol.

A 30-cm. silicic acid (Mallinckrodt, suitable for chromatographic analysis) column was prepared as described previously (*7*) except that 240 gm. of silicic acid and a 4-cm. diameter glass tube were used. After the column, covered with solvent, stood overnight, the butanol solution of the metabolites was carefully applied and washed twice with 3-ml. portions of butanol. The solvent reservoir was attached and 22-ml. fractions were collected each 100 minutes with gravity operation of the column. A 50-μl. aliquot of each tube was plated and counted in a windowless counter. The plot of radioactivity *versus* volume of eluate was similar in shape to that already described (*5*, *7*) except for altered values. The desired 1-OH- and 3-OH-2-FAA appeared together in the first peak with a volume of eluate of 180 to 880 ml. This peak contained 80 million cpm or 19 percent of the 424 million cpm recovered in all fractions. Similarly, 28 percent of 5-OH-2-FAA, 3 percent of 8-OH-2-FAA, and 30 percent of 7-OH-2-FAA were found at 1020 to 1400, 1440 to 1630, and 1660 to 2330 ml. of eluate, respectively.

The fraction containing the mixture of the 1-OH- and 3-OH-2-FAA was reduced to a small volume on a steam bath by a current of nitrogen. Amounts of 2.05 and 2.25 mg. of the unlabeled 1- and 3-hydroxy derivatives (*8*), respectively, were added as carriers, and the remaining solvent was removed. The residue was taken up in 2 ml. of *tert.*-butanol and rechromatographed on a 4-cm. diameter and 57-cm. high silicic acid column prepared with 464 gm. of silicic acid. The solution was washed with two 2-ml. aliquots of butanol, and the solvent reservoir was attached. Fractions of about 18 ml. of eluate were collected each 100 minutes by applying approximately 1-pound air pressure to the top of the column. Text-figure 1 shows the plot of radioactivity *versus* volume of eluate and other pertinent data. Over all, 97 percent of the radioactivity applied was recovered in the various fractions.

Fraction *c*, 22 million cpm or 28 percent of the activity applied, corresponding to 1-OH-2-FAA, was diluted with 45.6 mg. of the unlabeled carrier compound and taken to dryness in a stream of nitrogen. The residue was recrystallized twice from 50 ml. of 20 percent ethanol with 4 mg. of Norit. The product, 35.6 mg. of *N*-(1-hydroxy-2-fluoren-9-C^{14}-yl)acetamide, m.p. 208 to 210°, had a specific activity of 518,000 cpm per mg., and thus accounted for all of the radioactivity in fraction *c*.

Fraction *e*, 40 million cpm or 51 percent of the activity applied, corresponding to the 3-hydroxy derivative, was likewise treated with 59.3



TEXT-FIGURE 1.—Separation of *N*-(1- and 3-hydroxy-2-fluorenyl)acetamide by column chromatography. *See* text for conditions of experiment.

mg. of carrier. After 4 recrystallizations from 40 ml. of 25 percent ethanol, with 5 mg. of Norit on the first step, 40.8 mg. of material, m.p. 232 to 235°, was obtained. This substance was mixed with 40 mg. of compound obtained in a previous run (20 million cpm and 60 mg. of carrier) and recrystallized from 90 ml. of 12 percent ethanol giving 69.4 mg. of the final product, *N*-(3-hydroxy-2-fluoren-9-C^{14}-yl)acetamide, m.p. 232 to 234°, with a specific activity of 285,000 cpm per mg.

It can be noted that the specific activity obtained was appreciably lower than that calculated (500,000 cpm/mg.) from the total activity and the amount of carrier used. Only 42 percent of the activity under the peak, *i.e.*, 21 percent of the amount of radioactivity applied to the column, was accounted for by the 3-hydroxy derivative. Other isotope-dilution experiments on chromatographic fractions, with the mobility of 3-OH-2-FAA, isolated from rat and guinea-pig urine (*cf. 1*), have given similar results. This suggests that there exists an as yet unknown metabolite having the same mobility [7] as the 3-hydroxy compound under our chromatographic conditions. This material appears to be less acidic than 3-OH-2-FAA, for it was found in the course of these experiments that it extracted into ether from a 0.5 N potassium hydroxide solution, and could, thus, be separated from 3-OH-2-FAA.

Metabolism studies.—Suspensions of the labeled compounds in an acacia solution were prepared in such a concentration that rats could be given

[7] On columns over 150 cm. long this material was occasionally found in the 1-OH-2-FAA fraction.

the desired amount of material (*cf.* text-figs. 2 and 4, and table 1) in 1 ml. and guinea pigs in 2 ml. of the suspension by intraperitoneal injection. Three female Buffalo strain rats were used for each compound. One rat each was killed after 1, 2, and 7 days, respectively, by exsanguination through the abdominal aorta, under ether anesthesia. Two female NIH-bred stock guinea pigs likewise received injections of 1-hydroxy and 2 received the 3-hydroxy derivative and were similarly killed 1 day later. Upon autopsy selected tissues were removed, dried *in vacuo*, and analyzed for radioactivity (*9*, *10*). Liver proteins were isolated from the perfused livers and separated into a soluble and an insoluble (0.2 M acetate buffer, *p*H 5.0) fraction as described previously (*9*).

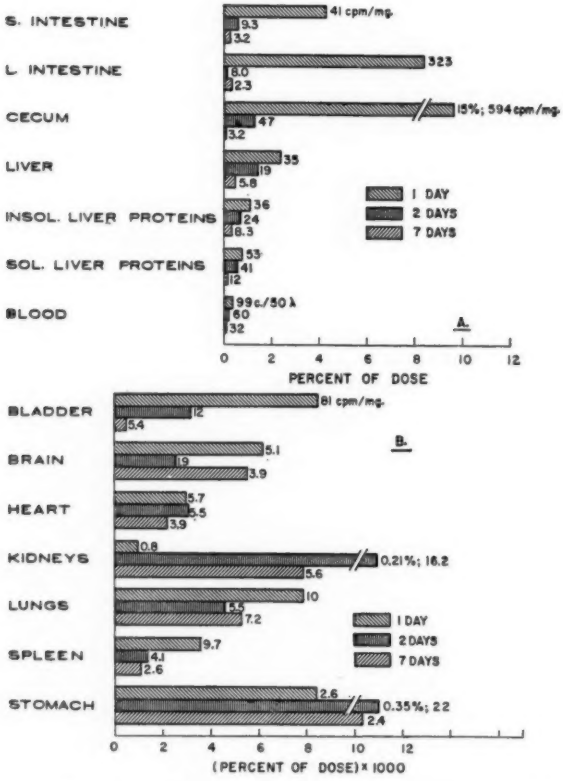
The urinary metabolites were resolved into the unconjugated, glucuronic acid and sulfuric acid conjugated fractions by a combination of ether extractions and enzymic hydrolyses. Paper or column chromatography was performed by our standard method (*7*) in 2 solvent systems: 1) Cyclohexane, *tert.*-butanol, acetic acid, and water (16:4:2:1 parts v./v.), and 2) *sec.*-butanol, 3 percent ammonium hydroxide (3:1). Samples of each fraction obtained by column chromatography were rerun on paper chromatograms for the purpose of correlating the 2 methods and also as a means of identifying each fraction.

In a number of instances the nature of selected chromatographic fractions was further established by a carrier-isotope dilution with unlabeled compound, or by ultraviolet and infrared spectroscopy (Cary recording spectrophotometer, model 14, ethanol solutions, or Perkin-Elmer recording spectrophotometer, model 21, solid potassium bromide disks, respectively). Sample preparation for the spectroscopic studies was performed by taking the chromatographic fractions to dryness *in vacuo*. The residue was taken up in a suitable volume of absolute ethanol for the ultraviolet spectrum. For the infrared spectrum the residue was dissolved in the smallest possible volume of ethanol which was mixed intimately with about 130 mg. of potassium bromide. The solvent was removed *in vacuo* in a desiccator, and the remaining solid was finely pulverized and worked up as usual for the preparation of the disk.

Results

Distribution and Excretion Studies

Distribution and excretion of radioactivity from N-(*1-hydroxy-2-fluorenyl*)-*acetamide in rats.*—Radioactivity was found in all organs and tissues analyzed (text-fig. 2). In most tissues the activity was highest after 1 day and declined rapidly thereafter. Much of the isotope was located in the intestinal tract—evidence of the rapidity of absorption and excretion of the compound and its metabolites. An appreciable proportion of the C^{14} in the liver was in the form of protein-bound radioactivity. While the insoluble proteins accounted for a larger fraction of this activity, the soluble proteins had a higher specific activity. There was no outstanding concentration of radioactivity in any one organ, though the blood carried substantial amounts of isotope. The slow decrease of the

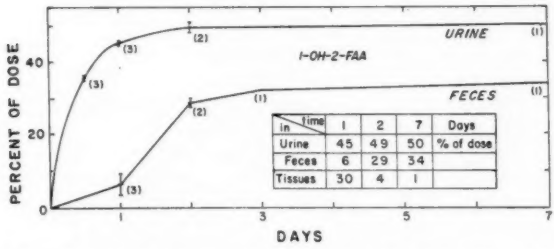


TEXT-FIGURE 2.—Distribution of isotope and specific radioactivity of dry rat tissues after the intraperitoneal injection of *N*-(1-hydroxy-2-fluoren-C^{14}-yl)acetamide. Each rat, weighing 120 gm., received 4.8 mg. of compound (1.68μmole/100 gm., 2.48 × 10^6 cpm). Tissues comprising the gastrointestinal tract were analyzed with the respective contents: *A*) tissues containing sizable amounts of radioactivity; *B*) tissues containing small amounts of radioactivity.

radioactivity in some organs, such as the brain or the lungs, as contrasted to the rapid increase and decrease of isotope in the tissues of the intestinal tract, is of some interest.

The excretion of radioactivity in the urine proceeded at a fairly fast initial pace (text-fig. 3). All 3 animals observed during the first day behaved in a similar manner in this respect. A total of 50 percent of the dose was eliminated in the urine in 7 days. Radioactivity appeared in the feces more slowly, but 34 percent of the dose was thus excreted.

Distribution and excretion of radioactivity from N-*(3-hydroxy-2-fluorenyl)-acetamide in rats.*—This compound was absorbed from the peritoneal cavity less readily than either 2-FAA or the 1-hydroxy derivative. Whereas there were no visible residues of the latter 2 compounds 24 hours after the doses given, some white radioactive particles were noted 24, and a



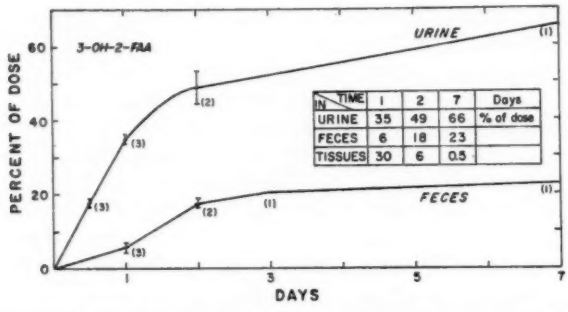
TEXT-FIGURE 3.—Excretion of radioactivity by rats after injection of *N*-(1-hydroxy-2-fluoren-C^{14}-yl)acetamide. Vertical bars indicate the standard error (*11*), and the figure in parentheses the number of rats. The data for tissues in the table are the sum of individual values of the organs analyzed (text-fig. 1). These exclude some tissues such as the carcass.

few even 48, hours after an injection of the 3-hydroxy derivative. Nevertheless, sizable amounts of radioactivity were found in all the organs analyzed (text-fig. 4). In fact, the specific activities of some of the tissues were of a similar order of magnitude as with the 1-hydroxy derivative, if the difference in specific activity of the compounds administered is considered. For example, the activity of the liver 1, 2, and 7 days after the injection of the 3-hydroxy derivative was 23, 11, and 3.5 cpm per mg. If these values are multiplied by 515,000/285,000, which is the ratio of the specific activities of the 1- and 3-hydroxy derivatives, respectively, the results obtained are fairly close to those observed with the 1-hydroxy compound, namely, 41, 20, 6.3 *vs.* 35, 19, 5.8. Thus, despite the relatively poorer absorption of the 3-hydroxy derivative, it gave rise to a number of tissue activities similar to, or even higher than, those seen after administration of the more readily absorbed 1-hydroxy derivative. It is interesting to note, however, the low activity of the blood and of the liver proteins observed with the 3-hydroxy derivative, as contrasted with the higher activities in these same tissues with the 1-hydroxy isomer.

The excretion of labeled material in the urine was somewhat slower with the 3- as compared to the 1-hydroxy derivative (text-fig. 5). Only 35 percent of the dose appeared in the urine after 1 day. However, by the 2d day, 49 percent, or as much as with the 1-hydroxy compound, was found. These results probably reflect the slower initial absorption of the 3-hydroxy compound. At the end of 7 days, 66 percent of the dose was excreted in the urine, and 23 percent was eliminated in the feces of 1 rat.

Comparative study of the pathway of radioactivity after intraperitoneal injection of N-*2-fluorenylacetamide,* N-(*1-hydroxy-2-fluorenyl*)*acetamide, and* N-(*3-hydroxy-2-fluorenyl*)*acetamide in guinea pigs.*—The metabolism of labeled 2-FAA in guinea pigs has been studied after oral administration (*1–3*), but data have not been available on the fate of the intraperitoneally injected compound. Therefore, for the purpose of uniformity and comparison with the present experiment on the *ortho*-hydroxylated derivatives, labeled 2-FAA was also injected into 2 guinea pigs (table 1). Absorption of this compound and of the 1-hydroxy derivative in the 1-day experi-

TEXT-FIGURE 4.—Distribution of isotope and specific radioactivity of dry rat tissues after the intraperitoneal injection of *N*-(3-hydroxy-2-fluoren-C^{14}-yl)acetamide. Each rat, weighing 120 gm., received 7.0 mg. of compound (2.45 μmole/100 gm., 2.0 × 10^6 cpm). Presentation of data as in text-figure 2.



TEXT-FIGURE 5.—Excretion of radioactivity by rats after injection of *N*-(3-hydroxy-2-fluoren-C^{14}-yl)acetamide. Presentation of data as in text-figure 3.

mental period was complete. However, some of the 3-hydroxy compound remained unabsorbed in the peritoneal cavity, as in the experiments with rats.

The radioactivity in the blood was quite low after injection of any of the 3 compounds. In the case of the guinea pigs injected with 2-FAA and with the 1-hydroxy derivative, the amount of radioactivity in the blood (2-FAA, 0.031% of dose; 1-OH-2-FAA, 0.07%) was much lower than that of rats (0.42 and 0.32% respectively) after the same time interval; with the 3-hydroxy derivative it was of a similar order of magnitude (0.12%) as in rats (0.08%). The fact that the bile of guinea pigs collected at autopsy carried a substantial quantity of isotope with all 3

TABLE 1.—*Comparative distribution and excretion of radioactivity 24 hours after intraperitoneal injection of labeled* N-*2-fluorenylacetamide,* N-(*1-hydroxy-2-fluorenyl*)-*acetamide, and* N-(*3-hydroxy-2-fluorenyl*)*acetamide into guinea pigs*

Guinea pig	Compound					
	2-FAA		1-OH-2-FAA		3-OH-2-FAA	
	A	B	C	D	E	F
Dose, mg	19. 8	19. 8	21. 85	14. 8	19. 55	19. 55
Dose, μmole/100 gm	2. 96	2. 86	2. 69	1. 94	2. 52	2. 45
Dose, million cpm	33. 2	33. 2	1. 86	7. 65	5. 55	5. 55
Blood,* percent of dose	0. 021	0. 038	0. 17†	0. 072	0. 11	0. 12
Bile,‡ percent of dose	0. 13	0. 31	0. 10	0. 16	—	0. 61
Liver, percent of dose	0. 15	0. 25	0. 45†	1. 3	0. 32	0. 25
Insol. liver proteins, percent of dose	0. 069	0. 065	0. 62†	0. 33	0. 14	0. 18
Sol. liver proteins, percent of dose	0. 036	0. 034	0. 24†	0. 12	0. 10	0. 11
Urine, percent of dose	58	55	45	61	25	31
Feces, percent of dose	20	0. 05	21	5	0. 4	0. 15
Feces, weight, gm	2. 136	0. 350	5. 860	1. 936	0. 836	0. 844

* Assuming a total blood volume of 23 ml. per animal (*18*). The activity was actually determined on 50-μl. aliquots.

† The 1-hydroxy derivative with a specific radioactivity of 85,000 cpm per mg. was in ected into this guinea pig. Owing to this low specific activity, some of the results, denoted by a dagger, were subject to rather large statistical errors which are inherent in the measurements of small quantities of radioactivity. Therefore, most of the discussion is based on the more reliable results obtained with guinea pig D. However, the data obtained with the lower specific activity material do not contradict but, in fact, support the results observed in the other experiment.

‡ Per ml. of bile. Actual data were obtained on 50-μl. aliquots.

compounds indicated that appreciable amounts of metabolites were being excreted by the liver. Considering the small amounts of radioactivity prevailing simultaneously in the blood of the guinea pigs, it would appear that metabolites are being removed more rapidly from the blood in guinea pigs than in rats.

The percentage of a dose found in the liver was similar in guinea pigs that received 2-FAA to those that received the 3-hydroxy derivative, despite the fact that the latter compound was absorbed with more difficulty and thus was available at lower effective concentrations. On the other hand, the 1-hydroxy derivative gave rise to higher levels of radioactivity in the liver. The radioactivity bound to the liver proteins was

highest in the animals given the 1-hydroxy derivative, somewhat lower in those injected with the 3-hydroxy compound, and quite small in the guinea pigs administered 2-FAA. The latter finding is in agreement with the results noted after oral administration (*1–3*).

The excretion of radioactivity in the urine of the guinea pigs injected with 2-FAA proceeded fairly rapidly. In the 24-hour experimental period an average of 57 percent of the dose was thus eliminated. This is somewhat less than that found in the urine of animals fed the compound orally (*1*). The urine of the animals injected with the 1-hydroxy derivative also accounted for a similar proportion of the dose (53%), but the animals given the 3-hydroxy derivative had much lower levels of activity (28%) in the urine, probably because of the slower absorption of the compound. The data on the excretion of radioactivity in the feces are heterogeneous with all the compounds studied, possibly as a result of the variations in bowel movements of the different animals (*cf.* table 1) in the relatively short experimental period.

Ether-extractable metabolites of N-(*1- and 3-hydroxy-2-fluorenyl*)*acetamide in the urine of rats and guinea pigs under various conditions.*—The compounds administered are preferentially ether soluble and would be expected to extract into ether from urine at *p*H 6. Most of the unconjugated metabolites of these compounds would probably behave likewise. Thus, ether extraction of the urines at *p*H 6 gives a measure of the free, unconjugated metabolites. These amounted to about 5 percent in rats, and 1 to 3 percent in guinea pigs with either 1-OH- or 3-OH-2-FAA (table 2).

Metabolites excreted as glucuronides were estimated by the amount of radioactivity extractable into ether after hydrolysis by β-glucuronidase. Fifty-nine percent of the metabolites of the 1-hydroxy derivative were

TABLE 2.—*Percentage of urinary radioactivity extractable into ether under various conditions*

Urine treatment	Percent of urinary radioactivity in ether layer			
	Compound			
	1-OH-2-FAA		3-OH-2-FAA	
	Rats	Guinea pigs	Rats	Guinea pigs
a) Buffer to *p*H 6	5. 6	2. 8	4. 6	1. 4
b) Aqueous from *a* incubated with β-glucuronidase for 24 hours	59	85	76	87
c) Aqueous from *b* incubated with Taka-Diastase for 24 hours	8. 3(22)	0. 6(5. 2)	0. 2(1. 1)	0. 6(5. 7)

a) The pooled urine of 3 rats and the combined urine of 2 guinea pigs was buffered to *p*H 6, and extracted 5 times with freshly distilled ether.

b) The ether was removed from the aqueous layer by a stream of nitrogen; bacterial β-glucuronidase was added and the solution was incubated for 24 hours, and extracted 5 times with ether.

c) *Idem* as after *b*, except for incubation with Taka-Diastase. Figures in parentheses represent the percentage of ether-extractable material based on the radioactivity present in the aqueous phase after operation *b*.

conjugated with glucuronic acid in rats; the proportion of glucuronides was somewhat higher, 76 percent, with the 3-hydroxy compound. In the latter case the hydrolysis with β-glucuronidase was more difficult. The incubation time had to be doubled and the amount of enzyme preparation increased over that customarily used in order to secure complete hydrolysis, which was determined by paper chromatography. Likewise, the complete removal of activity from the aqueous layer after completion of incubation was achieved only by 8 to 10 repeated ether extractions as compared to 5 used with the other compounds studied (*1*). In guinea pigs, glucuronides accounted for about 85 and 87 percent of the urinary radioactivity of the 1- and 3-hydroxy derivatives, respectively.

The conjugation with sulfuric acid was somewhat more dependent on the species and the compound (*cf.* table 2). Guinea-pig urine contained approximately equal amounts of these conjugates after the administration of either 1-OH- or 3-OH-2-FAA, calculated as a function either of the total urinary activity or of the isotope contained in the aqueous layer after performance of the previous operations. On the other hand, sulfate esters formed a larger share of the urinary radioactivity in rats after the 1- than after the 3-hydroxy derivative. Thus, the results on the amounts of free, glucuronic acid, and sulfuric acid conjugated metabolites of 1-OH- and 3-OH-2-FAA in rat and guinea-pig urine are similar to those noted with 2-FAA itself (*1*, *7*, *13*).

Identity of Urinary Metabolites

Paper Chromatography

Aqueous metabolites of N-(*1-hydroxy-2-fluorenyl*)*acetamide.*—Paper chromatography of the aqueous urinary metabolites of 1-OH-2-FAA in the butanol solvent system, followed by autoradiography, yielded 9 distinct spots with rat urine and 6 spots with guinea-pig urine (table 3). Spot #3 in the rat urine might actually represent 2 different compounds, as evidenced by their resolution in guinea-pig urine, owing presumably to the occurrence therein of different relative amounts. The spot with R_f 0.14 to 0.18 is the glucuronide of *N*-(1,7-dihydroxy-2-fluorenyl)acetamide,[8] and the other component of spot #3 (R_f 0.22–26) might be the glucuronide of the 1,5-dihydroxy derivative. Similarly, spot #5 might correspond to the sulfuric acid conjugates of these compounds. Spot #6 appears to be the glucuronic acid conjugate of 1-OH-2-FAA. Spots #8 and #9 seem to represent the free, ether-extractable metabolites, which are present in rather low concentrations in guinea-pig urine and were not revealed under the experimental conditions employed.

Aqueous metabolites of N-(*3-hydroxy-2-fluorenyl*)*acetamide.*—The urine of rats injected with 3-OH-2-FAA likewise gave rise to 9 spots representing metabolites (table 3). In this particular case there was a difference in the excretion of some of these metabolites related to the period of

[8] Our experience with the chromatographic behavior of metabolites of 2-FAA suggests these tentative assignments made on the basis of the mobilities of the compounds.

TABLE 3.—*Mobility and relative intensity of spots of urinary metabolites of* N-(*1-hydroxy-2-fluorenyl*)*acetamide and* N-(*3-hydroxy-2-fluorenyl*)*acetamide in rats and guinea pigs**

Mobility† and relative intensity‡						
N-(1-Hydroxy-2-fluorenyl)-acetamide			*N*-(3-Hydroxy-2-fluorenyl)acetamide			
Spot No.	Rats	Guinea pigs	Spot No.	Rats		Guinea pigs§
	0–12 and 12–24 hrs.‖	0–24 hrs.‖		0–12 hrs.‖	12–24 hrs.‖	0–24 hrs.‖
1	0–3 m	0–3 w	1	0–2 w	0–3 w	—
2	3. 5–8 m	5–8 w	2	—	16–21 s	10–28 m
3	15–26 s	14–18 s 22–26 t	3	23–24 m	—	—
4	32–37 w	—	4	34–41 s	—	—
5	47–50 s	45–47 t 48–51 m	5	45–50 s	45–50 s	61–65 s
6	52–61 s		6	52–58 w	52–58 w	—
7	73–77 w		7	69–75 w	66–71 m	74–77 w
8	81–86 m		8	79–85 m	78–83 m	—
9	91–95 m		9	87–91 m	—	—

*Aliquots of urines were applied to Schleicher and Schüll No. 598 paper strips (*1, 7*). The chromatogram was run in *sec.*-butanol, 3 percent ammonium hydroxide, 3:1 parts (v:v), and autoradiographs were made on Kodak No-Screen or Royal Blue X-ray film.

†Range (front to back of spot) of R_f values × 100.

‡Relative intensity designated s = strong, m = medium, w = weak, and t = trace.

§High salt content of urine and low radioactivity contributed to poor movement, resolution, and visibility of spots in this series of experiments.

‖Time of collection of urine sample. This factor had no effect on metabolites of 1-OH-2-FAA, which are therefore not shown separately. However, a difference between the urine collected at 0-12 hour and 12-24 hour periods was found, as shown, with 3-OH-2-FAA.

collection of the urine. Spot #2, tentatively assigned to the glucuronide of the 3,7-dihydroxy derivative, appeared in sizable amounts only in the urine collected 12 to 24 hours after the injection. On the other hand, two unknown components, spots #3 and #4, appeared mainly in the first 12-hour period. Spot #5 is believed to be the glucuronide of the 3-hydroxy derivative, and spots #8 and #9, near the solvent front, correspond to the free, ether-extractable compounds.

Resolution of the metabolites in the urine of guinea pigs receiving 3-OH-2-FAA was not as good as in rat urine, because of a fairly high salt content. It is, therefore, possible that some of the minor components did not appear and also that some of the mobilities shown were slightly altered under the experimental conditions present. Nevertheless, spot #2, due to the glucuronide of the 3,7-dihydroxy derivative, and spot #5, due to the glucuronide of the 3-hydroxy derivative, could be clearly distinguished.

Ether-extractable metabolites.—Paper chromatography was also routinely used to separate the metabolites contained in the various ether extracts, by means of the cyclohexane solvent system. In addition, paper chromatograms were run to check the identity of the various fractions obtained during column chromatography, as described later. These chromatograms

were helpful in revealing the nature and composition of a particular extract in a qualitative and sometimes semiquantitative manner.

Free, ether-extractable metabolites of N-(*1-hydroxy-2-fluorenyl*)*acetamide.*—In addition to some darkening at the origin, the chromatograms of extracts from rat urine showed spots of approximately identical relative intensities with R_f values of 0.00 to 0.03, 0.04 to 0.09, 0.08 to 0.20, and 0.58 to 0.74. Although authentic samples are not available, the nature of these and the other spots in this series of metabolites was deduced by means of the mobility and of the ultraviolet and infrared spectra of the larger samples of these compounds obtained in the column chromatographic procedure to be described. The first of the spots remains unidentified. The second spot appears to be *N*-(1,7-dihydroxy-2-fluorenyl)acetamide on the basis of the mobility on paper. For example, our previous work has shown that 2-FAA and the 1-hydroxy derivative had similar mobilities on paper in the solvent system used (*7*). Thus, it might be expected that further hydroxylation of both 2-FAA and the 1-hydroxy compound would give metabolites which likewise have similar mobilities. In this particular solvent system, 5-OH-2-FAA and 7-OH-2-FAA have R_f values of 0.12 to 0.20, and 0.04 to 0.10, respectively. The corresponding 1,5- and 1,7-dihydroxy derivatives were found to have R_f values of 0.08 to 0.20, and 0.04 to 0.09 (it is possible that the second hydroxyl group entered at the 4- rather than the 5-position, so that the spot with R_f of 0.08 to 0.20 would correspond to a 1,4-dihydroxy derivative). The fourth spot, R_f 0.58 to 0.74, contained 1-OH-2-FAA, but column chromatography suggested the presence of additional metabolites with similar mobilities.

The chromatograms of extracts from guinea-pig urine showed only one main component, the 1,7-dihydroxy compound. There was only a trace of the 1-hydroxy derivative.

Ether-extractable metabolites of N-(*1-hydroxy-2-fluorenyl*)*acetamide conjugated with glucuronic acid.*—After hydrolysis of the rat urine with β-glucuronidase, the ether extracts gave a chromatographic picture similar to that described for the unconjugated compounds. Further details and quantitative data on these conjugates were obtained by the column technique.

Paper chromatograms obtained in parallel runs with guinea-pig urine exhibited only one major spot corresponding to the 1,7-dihydroxy derivative.

Ether-extractable metabolites of N-(*1-hydroxy-2-fluorenyl*)*acetamide conjugated with sulfuric acid.*—After hydrolysis of both rat and guinea-pig urine with Taka-Diastase, the ether extracts were resolved into only 2 components. The dense spot, R_f 0.04 to 0.09, appeared to be the 1,7-dihydroxy compound. A faint radioactive area was near the origin. The possibility that other sulfuric acid conjugates might have been present, but were not hydrolyzed by Taka-Diastase, would, of course, also have to be considered. However, a number of experiments were performed in which the aqueous layer remaining after ether extraction

following the enzymic hydrolysis was adjusted to *p*H 1 and extracted continuously with ether. This procedure would be expected to split sulfuric acid conjugates chemically, and the free metabolites thus liberated should be found in the ether extracts. Nevertheless, no further metabolites were observed upon paper chromatographic examination.

Free, ether-extractable metabolites of N-(*3-hydroxy-2-fluorenyl*)*acetamide.*—Chromatography of the ether extracts of rat urine yielded 3 spots. The principal one, R_f 0.24 to 0.50, corresponded to the 3-hydroxy derivative itself. Relatively slight amounts of activity were associated with the spots of R_f 0.04 to 0.09 and 0.02 to 0.05 assigned to the 3,5- or (3,4-) dihydroxy and the 3,7-dihydroxy derivatives, respectively.

The ether extracts of guinea-pig urine were resolved into 2 spots. A rather faint area corresponded to the 3-hydroxy compound, and the other darker one to the 3,7-dihydroxy derivative.

Ether-extractable metabolites of N-(*3-hydroxy-2-fluorenyl*)*acetamide conjugated with glucuronic acid.*—The chromatograms of the ether extracts, obtained after enzymic hydrolysis of rat urine with β-glucuronidase, exhibited 3 spots, as observed with the free metabolites. Their relative proportion, however, was altered in that slightly more of the dihydroxylated derivatives seemed to be present.

In guinea-pig urine the same 2 components, found free, were also observed as the glucuronides. In this case there was a shift in the relative quantities present, for the spot due to the 3-hydroxy compound appeared to be of a magnitude similar to that of the 3,7-dihydroxy derivative. Quantitative data obtained by column chromatography (*cf.* a later section) bore out these semiquantitative impressions.

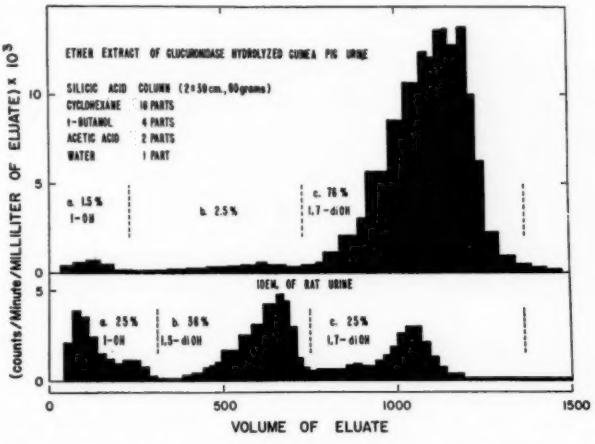
Ether-extractable metabolites of N-(*3-hydroxy-2-fluorenyl*)*acetamide conjugated with sulfuric acid.*—After incubation with Taka-Diastase, both rat and guinea-pig urine gave rise to a spot corresponding to the 3,7-dihydroxy derivative. In analogy to the other compounds of this series, conjugation with sulfuric acid seemed to occur only when a hydroxy group was present on the 7-position of the fluorene ring (*cf.* 7).

Column Chromatography

Separation of metabolites on a column was applied to the ether-extractable compounds after enzymic hydrolysis of the glucuronic acid conjugates. This fraction was the largest one (table 2), and it was therefore studied further in a system providing optimal resolution and at the same time allowing the collection of quantitative data on the composition of the mixture of metabolites.

Metabolites of N-(*1-hydroxy-2-fluorenyl*)*acetamide.*—Separation of the ether extract of rat urine on a 30-cm. silicic acid column provided 3 peaks, *a*, *b*, and *c*, accounting for 25, 36, and 25 percent, respectively, of the amount of activity applied (text-fig. 6). In contrast, the ether extract of guinea-pig urine contained 76 percent of the activity in the component corresponding to peak *c*. Each of the components was well separated from the adjoining material, and provided a sufficient quantity of each

fraction to permit carrier-isotope dilution experiments and to initiate spectroscopic studies. The mobilities on the column of peaks *b* and *c* were rather similar to those observed with 5-OH-2-FAA and 7-OH-2-FAA (*7*). Hence these peaks very likely are the 1,5- (or 1,4-) and 1,7-dihydroxylated derivatives. Spectroscopic evidence tended to confirm these assignments, which must, however, remain tentative until comparison with authentic samples can be made.



TEXT-FIGURE 6.—Eluate diagram obtained on a silicic acid column upon separation of the ether-extractable metabolites (after hydrolysis by β-glucuronidase) of *N*-(1-hydroxy-2-fluorenyl)acetamide in rat (*bottom*) and guinea-pig (*top*) urine. The extract from rat urine contained 1,940,000 cpm (equivalent to 3.7 mg. of compound), and that from guinea-pig urine, 4,450,000 cpm (equivalent to 8.6 mg. of the 1-hydroxy derivative).

Peak *a*, from the rat-urine extract, expected to contain the 1-hydroxy derivative, was subjected to a carrier analysis with the available unlabeled 1-hydroxy derivative, since the ultraviolet absorption spectrum did not quite correspond to that of the known compound. This procedure showed that only 20 percent of this peak was accounted for by 1-OH-2-FAA. A portion of this peak was, therefore, rechromatographed on a high silicic acid column whereby it was resolved into 6 components (table 4). Of these, fraction III showed the ultraviolet spectrum of authentic 1-OH-2-FAA. The lower value, 13 percent, found by this method as compared to the carrier analysis, 20 percent, already discussed, may be ascribed to the manner in which fractions under the peak were selected. In any case, the agreement between the data obtained by the 2 different methods is fairly satisfactory.

Metabolites of N-*(3-hydroxy-2-fluorenyl)acetamide.*—The principal component (69%) in rat urine was the 3-hydroxy derivative. The 3,5- (or 3,4-) and 3,7-dihydroxy compounds amounted to about 8 and 9 percent, respectively (text-fig. 7). On the other hand, guinea pigs excreted less

TABLE 4.—*Column chromatographic separation of peak* a *(text-fig. 6)**

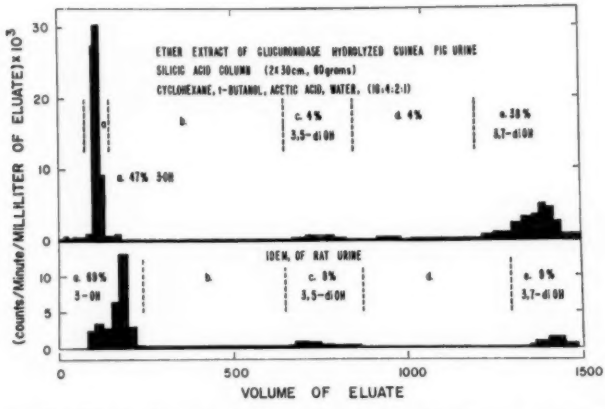
Fraction	Eluate volume *(ml.)*	Radioactivity cpm × 10^{-3}	Percent of total	Identity
I	0–600	31	11	—
II	600–700	100	35	—
III	700–920	38	13	1-OH-2-FAA
IV	1025–1245	55	19	—
V	1245–1500	36	12	—
VI	1550–1700	28	10	—

*A silicic acid column, 1.6 × 185 cm., was prepared with 185 gm. of silicic acid. The cyclohexane solvent system (7) was used. A portion (280,000 cpm) of peak a (*cf.* fig. 6, rat urine) in 0.2 ml. of solvent was applied to the column and elution of radioactivity was followed in the usual manner.

of the 3-hydroxy derivative (47%), and eliminated more as the 3,7-dihydroxy compound (38%). A small amount of the 3,5- (or 3,4-) dihydroxy derivative was also present. Despite the somewhat unsymmetrical shape of the peaks designated *a* on the chromatograms of the extracts of both rat and guinea-pig urine, it was demonstrated by carrier-isotope dilution and by spectroscopy that these peaks are entirely due to 3-OH-2-FAA.

These data support the conclusion that the 3-hydroxy derivative undergoes further hydroxylation to a lesser extent than the 1-hydroxy isomer in both rats and guinea pigs.

Just as the 1,5- and 1,7-dihydroxylated derivatives of *N*-2-FAA have mobilities similar to the 5- and 7-monohydroxylated compounds, the mobilities of the corresponding 3,5- and 3,7-dihydroxy isomers bear an identical relationship to the 3-hydroxy derivative (*cf.* text-figs. 6 and 7).



TEXT-FIGURE 7.—Eluate diagram obtained on a silicic acid column showing the separation of the ether-extractable metabolites (after hydrolysis by β-glucuronidase) of *N*-(3-hydroxy-2-fluorenyl)acetamide in rat (*bottom*) and guinea-pig (*top*) urine. The extract from rat urine contained 1,200,000 cpm (equivalent to 4.2 mg. of the 3-hydroxy compound), and that from guinea-pig urine, 2,400,000 cpm (or 8.4 mg. of compound).

Discussion

Few, if any, sensitive or specific color reactions or other suitable procedures for the determination of 1-OH-2-FAA and 3-OH-2-FAA or the corresponding amines in biological materials are available (*7*, *8*, *14*). Moreover, even if there existed analytical methods appropriate for the compounds administered, they most likely would not be applicable to the quantitative estimation of any metabolites that might be formed. Such disadvantages have been overcome by the use of compounds labeled in a stable position with readily detected and measured isotopic carbon.

The synthesis of the labeled 1-OH- and 3-OH-2-FAA by organic chemical methods would also be a formidable undertaking, and would be likely to result in rather low over-all yields. This difficulty has been avoided by isolating the desired *ortho*-hydroxylated derivatives by means of suitable chromatographic procedures from the urine of rats that received *N*-2-fluoren-9-C^{14}-ylacetamide. The products so obtained needed to be recrystallized several times to insure their chemical and radiochemical purity. This was done after the prior addition of at least an equivalent amount of the pure unlabeled compound to act as carrier. Owing to the dilution of the final labeled material with the carrier compound, it was necessary that the starting material used be of very high specific radioactivity (*5*) so as to obtain a product of adequate isotope content for metabolism studies.

The results described herein indicate that conjugation of the hydroxylated compounds with glucuronic acid took place to a greater extent in guinea pigs than in rats. On the other hand, less of the free, unconjugated metabolites were found in guinea-pig than in rat urine. Similar observations had been made with respect to the metabolites of 2-FAA and of 7-OH-2-FAA (*1*, *7*, *15*).

A further difference resides in the hydroxylating ability of guinea pigs and rats toward these fluorene derivatives. The present as well as the previous (*cf. 1*) investigations indicated that the principal site attacked by guinea pigs was the 7-position, while rats hydroxylated a number of the carbon atoms of the fluorene ring system. The hydroxylation took place even though the molecule already contained a hydroxy group. However, further hydroxylation of 3-OH-2-FAA seemed to occur to a smaller extent than that of 1-OH-2-FAA, even in guinea pigs. An analogous situation was noted with the 7-hydroxy derivative (*15*). The extent of dihydroxylation, or the facility with which a second hydroxy group enters a given molecule, may depend, among other factors, on the chemical structure of the fluorene derivative, and in particular on the position occupied by the first phenolic function.

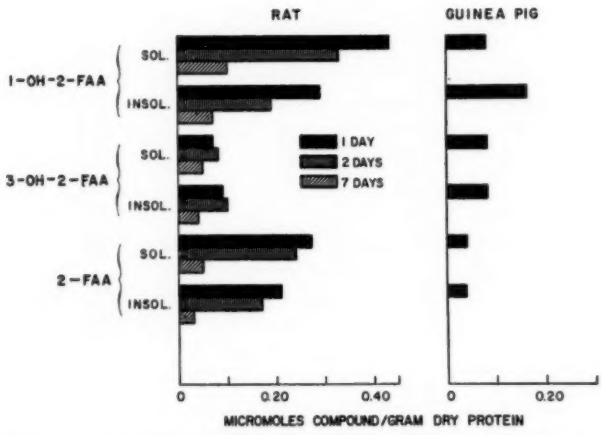
It should also be emphasized at this point that the identity of the polyhydroxylated derivatives described here is tentative, being based on the mobility of the compounds on paper and column chromatograms and on certain spectral features observed on the chromatographic fractions. While it is believed that the structures so deduced are correct, positive proof of their identity must await a comparison with pure samples ob-

tained by synthesis, or the isolation of sufficient material for purification, analysis, and degradation.

The question arises whether the difference in metabolism between rats and guinea pigs is related to the pronounced difference in the amount of protein-bound radioactivity in the livers of animals receiving injections of 2-FAA, and the 1- and 3-hydroxy derivatives (text-fig. 8). In all but one case the compounds studied gave rise to lower activities in guinea pig than in rat proteins. The exception was the soluble proteins of guinea pigs administered 3-OH-2-FAA which were, however, but a trace higher than their rat counterparts. An extreme case in the trend toward lower binding in guinea-pig proteins was reported recently with the 7-hydroxy derivative (*15*). In that instance rat liver proteins bound as much or more isotope as after a comparable dose of 2-FAA, whereas guinea-pig liver proteins carried no radioactivity (*15*).

The drop in radioactivity of the soluble and insoluble rat liver proteins, as a function of time, occurred with a half life of 3 to 4 days with 1-OH-2-FAA and 2-FAA. This corresponds to the rate of disappearance of protein-bound azo dye, which also had a half life of 3 to 4 days (*16*). The proteins from rats injected with 3-OH-2-FAA did not seem to follow this pattern.

Another dissimilarity between the rat and the guinea pig is the specific activity of the soluble as compared to the insoluble liver proteins. Thus, the soluble and insoluble proteins of guinea pigs showed a similar level of radiocarbon after a dose of either 2-FAA or the 3-hydroxy derivative; after the 1-hydroxy derivative the insoluble proteins exhibited a higher concentration of the label. In rats, on the other hand, the soluble proteins had the higher specific activity after 2-FAA and the 1-hydroxy derivative,



TEXT-FIGURE 8.—Comparison of the specific radioactivity, calculated and expressed in μmole of compound per gm. of dry protein, of the soluble and insoluble liver proteins of rats and guinea pigs injected with *N*-(1-and 3-hydroxy-2-fluorenyl)acetamides, and with *N*-2-fluorenylacetamide.

and slightly lower values after the 3-hydroxy compound. The interpretation of these observations is difficult at this time, but they may have some significance in terms of the types of proteins involved in the binding, or perhaps as a function of the metabolites present in the liver. Likewise, the question as to whether guinea-pig livers have the proteins required for binding as rats do cannot be answered now. It is true that more activity was bound to the insoluble guinea-pig liver protein and both soluble and insoluble rat liver proteins after administration of 1-OH-2-FAA than with any of the other compounds, which tends to focus special interest on this compound. However, the quantity bound, after injection of 1-OH-2-FAA into guinea pigs, is not equivalent to that present in rats, which may be attributed to any number of factors (permeability of cell membrane to compound, rate of protein synthesis, etc.). There is also no explanation for the curious inversion of activity bound to soluble as compared to that bound to the insoluble proteins. Further work on, and perhaps a different approach to, the fundamental problem of the resistance of guinea pigs to carcinogenesis by 2-FAA seems to be in order.

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An Autoradiographic Study of Tumor Dynamics[1]

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Summary

Autoradiograms of sections of tumors labeled *in vivo*, by giving a single dose of a labeled amino acid to the host, were examined for the pattern of uptake. Two rat tumors and 2 mouse tumors were investigated with glycine-1-C^{14} and L-lysine-U-C^{14}. Similar results were obtained with all combinations. No gradients of isotope concentration are observable in viable tissue at any time from 7 hours to 2 weeks after the administration of amino acid. Necrotic tissue does not fix isotope, and labeled tissue on becoming necrotic releases isotope only very slowly. The observations as a whole suggest that tumor proteins are not distinguished by a non-dynamic character, and that peripheral growth is not a factor of particular significance in the propagation of tumors.—**J. Nat. Cancer Inst. 22: 845–867, 1959.**

Among questions of continuing interest in connection with the growth of tumors are several which concern the supply of nutrients and their pattern of distribution and utilization within a tumor. It is sometimes suggested, for example, that tumors grow more rapidly, or exclusively, at the periphery; that circulation becomes inadequate at the center and its failure is the cause of necrosis; that the host is depleted by unsuccessful competition for circulating nutrients; and that tumor proteins, being unavailable to the host for its metabolic needs, are distinguished by a negligible participation in the "dynamic equilibrium" of Schoenheimer. This communication describes an attempt to visualize these dynamic factors by means of autoradiography.

The experimental procedure consisted essentially of administering a single dose of a radioactive amino acid to a tumor-bearing host, subsequently killing the animal, slicing the tumor in half, and preparing an autoradiogram from the cut face. By suitable variations of tumor age and the time intervals allowed for isotope uptake, it was expected that different features of the isotope incorporation would be made visible. For example, an experiment of brief duration in which the specific activity

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of the tumor proteins would be increasing rapidly (*1*) might reveal preferential uptake at the periphery in the form of a specific activity gradient decreasing in intensity from the outside inward, and/or might demonstrate something of the pattern of circulation. A longer period of uptake might show a gradient outward as new tissue of gradually decreasing specific activity was laid down. Alternatively, absence of gradients might be observed which might indicate mobility of the bound isotope. In addition, the metabolic behavior of areas of necrosis and incipient necrosis should be evident.

Procedure

Tumors.—Four tumors of different types were used: the Walker carcinosarcoma 256, a rapidly growing tumor carried subcutaneously in Sprague-Dawley rats; the ACMCA2 fibrosarcoma,[3] a more slowly growing tumor carried subcutaneously in Irish rats; the C3HBA mammary adenocarcinoma,[3] a moderately rapidly growing tumor carried subcutaneously in C3H/An mice; and the S91 melanoma,[4] a rapidly growing tumor carried intramuscularly in C × DBA mice. All tumors had been propagated for at least several score of generations.

With the exception of the Walker, the tumors were selected on the basis of minimal tendency to necrosis. The best in this respect is the melanoma, which was transplanted as a cellular dispersion and exhibited only an occasional microscopic region of focal necrosis. The other tumors were transplanted as single fragments. The adenocarcinoma developed moderate diffuse necrosis, while both rat tumors developed a grossly necrotic center surrounded by a viable shell. The necrotic region was considerably less extensive in the fibrosarcoma than in the Walker tumor, and the viable shell was thicker.

Dosage.—Two amino acids were used: glycine-1-C^{14} and L-lysine-U-C^{14}. The dosage protocols are tabulated in table 1. The mass of amino acid, in general, was of the order of 10 to 20 mg. per kg. for glycine and 2 mg. per kilo for lysine, which is considered to be within the tracer range. The amino acids were administered in aqueous solution by stomach tube and the animals were maintained on chow *ad libitum*.

Handling of specimens.—In most cases, the freshly excised tumor was sliced through the center, and the autoradiogram was made from the cut face. With large tumors, a second cut was made parallel to the first to remove excess tissue and facilitate subsequent fixation. The specimen then consisted of a thick (1 cm.) slice, and was fixed in Zenker-formol solution for 6 hours, which appeared to be sufficient even for large slices. (Tellyesniczky's fluid works equally well.) The fixative was thoroughly washed out for 48 hours, and the specimen was embedded in paraffin. The block was placed in a microtome, with the center face out, and carefully shaved until a perfectly smooth surface was obtained. A section was saved for subsequent staining with hematoxylin and eosin and for microscopic examination. It was necessary to dehydrate the larger blocks

[3] Supplied by Dr. Morris K. Barrett.
[4] Supplied by Mr. Richard V. Eck.

TABLE 1.—*Experimental protocols*

Experiment No.	Tumor	Weight of host + tumor at injection (*gm.*)	Weight of tumor at death (*gm.*)	Tumor age at injection (*days*)	Duration of expt.	Tracer	Dose (*μc*)	Dose (*mmole/kg.*)	Remarks
1	Walker	257	*ca.* 6	14	7 hours	Glycine	20	.15	Tumor in good condition except for hemorrhagic spot. Longitudinal and equatorial cuts made.
2	Walker		1	7	24 hours	Glycine	21		No necrosis. Longitudinal and equatorial cuts made.
3	Walker	345	58.5	37	72 hours	Glycine	60	.35	Tumor grossly necrotic but little fluid present. Longitudinal and equatorial cuts made.
X	Walker	320	39.5	37	10 minutes	Glycine	130	.82	Extensive central necrosis. No image on autoradiogram.
5	Walker	206	67	15	11 days	Glycine	46	.45	Extensive central necrosis. Tumor increased *ca.* 7× during expt. Rat gained 35 gm. during expt.
7	Walker	281	10.0	20	7 hours	Lysine	45	.018	Extensive central necrosis.
8	Walker	230	47.6	6	12 days	Lysine	50	.024	Extensive central necrosis. Some fluid. Rat gained 50 gm. during expt.
9	Walker	235	19.0	13	5 days	Lysine	50	.024	Rat gained 34 gm. during expt.
10	Walker	235	12.0	6	12 days	Lysine	50	.024	Central necrosis, not liquid. Rat gained 78 gm. during expt.

See footnotes at end of table.

TABLE 1.—*Experimental protocols*—Continued

Experiment No.	Tumor	Weight of host + tumor at injection (*gm.*)	Weight of tumor at death (*gm.*)	Tumor age at injection (*days*)	Duration of expt.	Tracer	Dose (*μc*)	Dose (*mmole/kg.*)	Remarks
11	Walker	243	34. 2	13	5 days	Glycine	64	. 55	Central necrosis, much liquid. Rat gained 31 gm. during expt.
12	Walker	231	26. 5	6	12 days	Glycine	64	. 56	Central necrosis, not liquid. Rat gained 53 gm. during expt.
13	ACMCA2	214	6. 6	11	14 days	Glycine	44	. 15	Central necrosis.
14	ACMCA2	206		11	14 days	Lysine	45	. 023	Central necrosis. Rat lost 6 gm. during expt.
15	ACMCA2	212		25	7 hours	Glycine	43	. 16	Central necrosis.
16	ACMCA2	221		25	24 hours	Glycine	43	. 15	Central necrosis.
17	ACMCA2	224	30	25	13 days	Glycine	43	. 15	Central necrosis. Rat gained 3 gm. during expt.
18	ACMCA2	231		25	7 hours	Lysine	45	. 016	Tumor ulcerated, central necrosis.
19	ACMCA2	208		25	24 hours	Lysine	45	. 018	Central necrosis.
20	ACMCA2	228	45. 5	25	13 days	Lysine	45	. 016	Central necrosis. Rat gained 16 gm. during expt.
21	S91	26. 5	*	9	14 days	Glycine	4. 7	. 21	
22	S91	23. 5		9	6 days†	Lysine	5. 0	. 018	
23	S91	26. 5		19	7 hours	Glycine	4. 7	. 21	

24	S91	25. 0		19	25 hours	Glycine	4. 7	. 22	Very little necrosis occurred in the S91 preparations.
25	S91	28. 5		19	10 days	Glycine	4. 7	. 19	
26	S91	27. 0		19	7 hours	Lysine	5. 0	. 016	
27	S91	26. 0		19	25 hours	Lysine	5. 0	. 016	
28	S91	28. 7		19	10 days	Lysine	5. 0	. 015	
30	C3HBA	31. 2	3. 0	15	7 hours	Glycine	4. 7	. 18	
31	C3HBA	21. 3	0. 4	15	25 hours	Glycine	4. 7	. 26	
32	C3HBA	27. 5	5. 3	15	6 days	Glycine	4. 7	. 20	Mouse gained 4.0 gm. during expt. Tumor diffuse.
33	C3HBA	22. 0	4. 0	15	10 days	Glycine	4. 7	. 025	Mouse gained 4.0 gm. during expt.
34	C3HBA	27. 0	1. 7	15	7 hours	Lysine	5. 0	. 016	Very little necrosis.
35	C3HBA	26. 4	2. 1	15	25 hours	Lysine	5. 0	. 020	Necrotic center.
36	C3HBA	26. 0	5. 2	15	10 days	Lysine	5. 0	. 019	Mouse gained 1.4 gm. during expt.

*The S91 is too fragile to excise. The entire hind leg was taken and fixed, the bone was decalcified in formic acid-sodium citrate, and the specimen was sliced longitudinally after embedment.
†Animal died prematurely at 6th day.

thoroughly for 2 weeks before embedding them, or otherwise residual moisture remained and evaporation from the specimen caused the cut surface to become concave. Close contact with the photographic emulsion then could not be obtained, and the image was not sharp.

In experiments 1 and 2, the tissue specimens were each sandwiched before fixation between 2 stainless steel screens held by rubber bands. This was done to avoid the possibility that the rather thick specimens might curl, but the soft tumor tissue extruded readily through the mesh and a heavy imprint of the screen remained in the block, which appeared in the autoradiograms and made their interpretation difficult. The practice was therefore abandoned. Curling, fortunately, did not prove to be troublesome.

Autoradiography.—All the tissue blocks were placed face down on a single large sheet of Eastman No-Screen X-ray film, with a brass weight on each to insure good contact. The record so produced is limited by the range of the C^{14} *beta* particle to a tumor section no more than one-quarter of a millimeter thick, and, since the thickness of the tissue block exceeded this saturation thickness, the exposure received by the film was proportional to the specific activity of the tissue. After 15 days' exposure the film was developed 5 minutes in D-11 at 68° F. in a tray, with continuous agitation.

Blank experiments made with unlabeled tumor specimens verified that no artifact image was produced by chemical effect of the tissue, fixative, or paraffin. When the single-film exposure of the labeled blocks was made, however, a faint image of the paraffin block appeared around several specimens. Preliminary exposures of all the blocks had been made earlier without the appearance of this effect, but check exposures made subsequently from one of the blocks without the use of a brass weight also gave the effect. The cause of the effect was not investigated further, but a possible explanation may lie in the observation that, in the single-film exposure, some of the blocks had adhered weakly to the emulsion, while others were entirely free. It is thought that static electricity, generated when the adhering blocks were broken loose, may be responsible for the artifacts.

Free glycine appears to be removed in the preparation of the specimens. This can be seen from experiment X, table 1, in which a tumor was taken after 10 minutes' uptake of glycine. Although at this time the specific activity of free glycine in the plasma should already be very substantial (*2*), and although a heavy dose of tracer was given, no image appeared on the autoradiogram. Thus the autoradiograms are essentially records of bound amino acid which is, of course, mainly in the form of protein.

For the preparation of the illustrations which accompany this paper, the single film bearing all the autoradiograms was photographed and a print was made the same size as the original. This print was then cut to prepare the plates, which are also reproduced 1:1. Thus the illustrations show the actual size of the tumors and also give an indication of the densities of the autoradiograms. Unfortunately, considerable compression

of the tonal scale occurs during reproduction so that the attempt to maintain a semblance of the original density relationships causes the darkest prints to be blocked up and it is difficult to see the detail in the lightest ones. The original autoradiograms were not blocked up, however; photoelectric scanning through a pinhole-size aperture revealed no density greater than 1.1, which is a value well within the usable portion of the sensitometric curve. The originals exhibited no features not adequately represented by the reproductions.

Results and Conclusions

Initial Experiments With the Walker Tumor

Figure 1 shows the autoradiogram of a rather young tumor after 7 hours' uptake of glycine, and one of the tissue blocks from which it was made. A moderate concentration of isotope is present. There is no evidence of a gradient, though the pattern is somewhat irregular. No interpretation is assigned to the irregularity because of a complication which arose in this first experiment from the use of the stainless steel screens mentioned earlier. These blocks were recut to try to remove the screen pattern but the effort was not entirely successful, and so much was removed from the blocks that it is not certain they exceed the saturation thickness at all points.

Necrosis was just beginning in this tumor and is visible as a hemorrhagic spot in the tissue block. This spot was surrounded by tissue before the block was cut back and showed little or no activity in a preliminary autoradiogram, demonstrating that the tissue loses its ability to synthesize protein when it becomes necrotic. This feature is not readily apparent in figure 1 but is evident in subsequent experiments.

To eliminate necrosis altogether, a very small tumor was used (fig. 2), and the experimental interval was increased to 1 day to allow a little growth to occur. The autoradiogram does not reveal any evidence of nonuniformity in the uptake pattern. The specimens are rather small for this to be apparent, but photographic enlargement of the images (not shown) does not reveal any additional features.

A large tumor was used in the next experiment (fig. 3), and the period of incorporation was extended to 3 days. This tumor was grossly necrotic and the metabolic inertness of the necrotic area is clearly evident. The uptake pattern in the viable area does not suggest the appearance of a gradient. The irregularity in the boundary region of the necrotic zone can be satisfactorily ascribed to intercalation of viable and necrotic tissue in the boundary of the necrotic zone (*see* photograph, fig. 4).

In all the experiments just described, the tumors were cut both longitudinally and transversely to check on the possibility of different degrees of uptake in different directions, perhaps due to the circulatory pattern. Since no such effect was observed, this practice was discontinued, and in all subsequent experiments a single longitudinal cut was made.

In the final experiment of this group a young tumor was used in which

necrosis would be either absent or just beginning, and the uptake period was extended to 11 days. The autoradiogram is shown in figure 5 and a stained section of the tumor is shown in figure 6. In this case the outer zone is uniformly labeled and a region of more highly labeled material is present within the necrotic area. This outcome is interpreted to mean that the highly labeled material was not necrotic when the tracer was administered and became labeled in the usual fashion, but became necrotic during the first 2 or 3 days while still highly radioactive. The isotope was then locked into the now metabolically inert material at this level, which is higher than that of tissue laid down later when the specific activity of the tracer has fallen to a much lower level. It will be observed that this is a double tumor and that the specific activity of the necrotic material is greater in the larger nodule than in the smaller. This is to be expected, since the smaller nodule was presumably smaller also at the time of glycine administration, and the onset of necrosis would occur in it later. This would allow the specific activity of the labeled tissue to drop to a lower level before being halted by tissue death.

Around the edge of this autoradiogram, as well as several of the others, can be seen a discontinuous line of slightly higher density than the interior. We are not certain what this represents but believe it is probably the image of the membrane which surrounds many of these tumors.

In a few experiments the initial weight of the tumor is small enough and the experimental period is long enough to permit a determination of whether the host itself has experienced a gain or loss of weight during the experiment. Experiment 5 is one such case. During the growth of a tumor 15 days old at the beginning of the experiment, the tumor attained a weight of 67 gm., while the weight of the host plus tumor increased only 35 gm. (table 1). Since a 15-day-old tumor weighs only about 6 gm. (*cf.* expt. 1), it is clear that the host itself has experienced a net loss of weight. This phenomenon occurs generally with large Walker tumors, though not with smaller ones (*3*).

Confirmation With Lysine

Before proceeding further, 2 of the experiments described earlier were repeated with lysine to gain assurance that the behavior observed was not in some way unique to carboxyl-labeled glycine. Figure 7 shows a tumor of moderate size with a necrotic center permitted to take up isotope for 7 hours. Like glycine, the lysine is uniformly distributed in the viable shell and is taken up to a relatively slight extent by the necrotic material. Figure 8 shows a very small tumor, young enough to be free of necrosis at the time of injection, after taking up isotope for 12 days. The distribution of isotope is essentially uniform, resembling that of the smaller nodule of figure 5. There is only a slight indication of more highly labeled material within the necrotic zone, which indicates that the tumor had passed through the initial highly labeled phase before being overtaken by necrosis.

Further Observations on Necrosis

Two experiments were done with necrotic tumors of moderate size which were allowed to take up the tracer for a period (5 days) sufficiently long to permit observable growth. Glycine was used in one (fig. 11) and lysine in the other (fig. 9). The centers of these tumors were in poor condition at autopsy and a few fragments dropped out when the second cut was made. The structure remained sufficiently intact, however, to exhibit a ring of highly labeled material, which delineates a central area of low isotope concentration and an outer area of intermediate level.

This is interpreted to mean that, when the tracer was given, the necrotic area incorporated little or no isotope, and the viable area became highly labeled as usual. The necrotic front, advancing steadily outward, caused further tissue death, and that portion which was caught during the first 2 or 3 days remained highly labeled. Of the remaining peripheral area, some had fallen to a lower specific activity before being overtaken by necrosis, and the remainder was tissue formed from tracer whose specific activity had also fallen.

The hosts gained weight in all these experiments except experiment 11, in which there was probably little change.

The autoradiograms of figures 10 and 12 are essentially a check with lysine and glycine, respectively, on the behavior of a tumor small enough so that it has passed through the initial highly labeled phase before necrosis sets in.

It is apparent in all these experiments that a considerable and rather rapid, specific activity swing occurs between the highly labeled early phase and the weakly labeled late one, yet at no time do any features appear within the viable tissue which permit one to distinguish between older protein and the newly formed. Either the proteins are being laid down with an average specific activity, which is uniform across the viable area, or, if the specific activity is nonuniform at the time of incorporation, the isotope must be readily mobile so that any gradients are rapidly obliterated.

Experiments on Fibrosarcoma ACMCA2

To test the generality of the behavior described earlier, the experiments were extended to this tumor which grows more slowly than the Walker tumor and is carried in a different strain of rats. Figures 15, 16, and 17 show the result of giving glycine to rats which bear tumors of moderate size and allowing uptake to proceed for 7 hours, 24 hours, and 13 days, respectively. As before, the necrotic center is inert, and distribution of isotope in the viable peripheral area is essentially uniform if allowance is made for the irregularity of the pattern of necrosis. During the 13-day period it is evident that the areas which were initially highly labeled have lost considerable isotope, but the presence of comparatively highly labeled necrotic material is clearly visible. The host lost weight over the 13-day period.

Figures 18, 19, and 20 show the same experiment repeated with lysine, with the same result. Again the host lost weight over the 13-day period.

Figure 13 shows a glycine experiment on a tumor which was small enough to be non-necrotic at the time of isotope administration and was allowed to grow long enough for necrosis to begin. A uniformly labeled tumor is the result, which is interpreted, as before, to mean that the tumor had passed through the highly labeled stage before necrosis set in. Figure 14 shows the same experiment repeated with lysine and the same outcome. The clear, crescent-shaped area is an artifact caused by a fragment dropping out of the necrotic area when the tumor was sliced.

Experiments on the S91 Melanoma

In these experiments the observations were extended to an additional tumor in a different species. An interesting feature of the melanoma preparation is that the tumor is seen along with the contiguous muscle tissue. There is no visible indication in the interfacial zone that the tumor is starving the muscle.

Figures 23, 24, and 25 show the result of a glycine experiment in which tumors of medium size were allowed to take up isotope for periods of 7 hours, 25 hours, and 10 days, respectively. The specific activity of the tumor at 25 hours is at least as great as at 7 hours, and is much higher than that of the surrounding muscle. At 10 days, however, the tumor exhibits a specific activity only slightly greater than that of surrounding muscle. The uptake pattern is difficult to characterize, partly because of its irregularity and partly because of the small size of these preparations. Examination of stained sections, however, indicates that the irregularity is associated with the existence of open spaces within the tumor, and the impression one gets is that, within areas of uniform texture, the distribution of isotope is uniform. A photograph of a stained section is shown for comparison in figure 29.

Figures 26, 27, and 28 show the result of repeating the same experiment with lysine; the outcome is the same as with glycine.

Figure 21 shows the uptake of glycine, after 14 days, by a tumor which was very small at the time of injection. At 14 days, the specific activity of the tumor has fallen below that of muscle, indicating that during the later stages the tumor is building tissue from precursors whose over-all specific activity is less than that of the mouse carcass. Figure 22 is the same experiment repeated with lysine. Unfortunately, this mouse died from unknown causes on the 6th day. The specimen is of interest as an indication that the specific activity of the tumor was still high at this time.

Experiments on the Mammary Adenocarcinoma

This series of experiments extends the observations to an additional mouse tumor of a different type (carcinoma). Figures 30, 31, 32, and 33 show rather small tumors, at 7 hours, 25 hours, 6 days, and 10 days, respectively, after glycine administration. Figures 34, 35, and 36 show uptake, at 7 hours, 25 hours, and 10 days, respectively, after lysine adminis-

tration. No differences are apparent between the 2 amino acids. This tumor is rather diffuse and somewhat necrotic, and it is difficult to characterize the isotope distribution pattern in clear-cut terms. The persistence of a high specific activity for at least as long as 1 day is once again apparent, and a much lower level and a comparatively uniform distribution are seen later. The comparatively highly labeled material seen in the central area in the 6- and 10-day experiments is necrotic. In the experiments which ran 6 days and longer, the mice appear to have been losing weight from their tissues, though uncertainty of the initial tumor weight is too great to be positive of this.

Discussion

The uniformity of the results obtained with this reasonably representative group of tumors shows that the observations apply fairly generally.

The absence of observable gradients in the viable tissue at 7 hours can be interpreted to mean either that such gradients do not occur and presumably the nutrients are supplied by an ordinary diffuse type of circulation, or that growth is predominantly peripheral but the rate of the isotope distribution through the tumor after an initial fixation at the margin is rapid relative to the rate of change of specific activity of newly formed protein. The latter rate is rapid for the first 3 hours (*1*) so that either interpretation leads to an impression of a reasonably uniform rate of isotope incorporation through the body of viable tissue. In addition, the second interpretation requires that the bound isotope be readily mobile. Since the experimental period is short, encompassing the rising phase of the tissue-specific-activity curve, "mobility" is presumably synonymous with metabolic lability in the Schoenheimer sense, as distribution of isotope by mechanisms such as simple translocation of labeled cells or mitosis should be minimal and should, moreover, operate in the outward direction.

The question of the degree to which tumor proteins are metabolically labile is of interest in view of the well-known inability of the host to utilize the proteins of its tumor under starvation conditions, and a number of tracer studies dealing with this question have been reported.

Babson and Winnick (*4*) found that labeled Walker transplants, growing in an unlabeled host, lose isotope with an apparent half-time of about 5 days. Moldave (*5*, *6*), working with labeled nongrowing Ehrlich ascites cells *in vivo*, found that such cells lose isotope even more readily from protein and other cellular components as well as fix isotope when unlabeled nongrowing cells are implanted into a labeled host. Greenlees and LePage (*7*), working with implanted labeled TA3 ascites cells, found a loss of activity of about 9 percent per day and observed incorporation of isotope into carbon dioxide and into viscera of the host. Growing in the solid form, however, the TA3 tumor was found to increase steadily in total isotope content up to 4 days after administration of labeled glycine, as did the 15091A mammary adenocarcinoma. LePage *et al.* (*8*) had observed earlier that Flexner-Jobling implants increase in total isotope

content up to 6 days after a dose of labeled glycine, and had taken this as evidence of a one-way passage of nitrogen into this tumor. A primary mammary adenocarcinoma, however, failed to exhibit such isotope accumulation upon transplantation, though some tendency to do so appeared to develop in later generations (*7*).

It seems quite possible that retention of isotope for 6 days, by a rapidly growing tumor like the Flexner-Jobling, may be due to the phenomenon illustrated in figures 9 and 11, in which a rapidly advancing necrotic front locks a considerable amount of isotope into highly labeled protein laid down during the first day or two of the experiment. It therefore appears that there is no convincing evidence to support the idea that tumor proteins are metabolically inert, and there is considerable evidence which bespeaks the contrary. This would leave open the question of peripheral growth. Actually, however, it is difficult to explain the uniform labeling, observed in the short experiments, in terms of redistribution from the periphery by dynamic exchange when one recalls that apparent half-times of exchange process are typically of the order of days. For the same reason one might expect that, in the longer experiments, too, one would observe some indication of a gradient, if growth were predominantly peripheral. Of course, the larger tumors consist of a relatively thin shell of viable tissue surrounding an inert mass (*cf.* fig. 7) and, in a trivial sense, growth of such a tumor is necessarily "peripheral."

We therefore favor the first of the alternate interpretations mentioned earlier in which the supply of nutrients is considered to be essentially diffuse, whatever its exact pattern, and protein synthesis and presumably growth to be occurring at a uniform rate throughout the viable tumor mass. Gradients within microscopic regions are not excluded, but there is no reason to suppose that the existence of these would be peculiar to tumors.

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PLATES

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PLATE 77

Uptake of glycine by Walker tumors.

FIGURE 1.—Small tumor after 7 hours' uptake. Autoradiograms of equatorial (*left*) and longitudinal sections, and (*below*) a photograph of equatorial tissue block. *Arrows* indicate hemorrhagic spot.

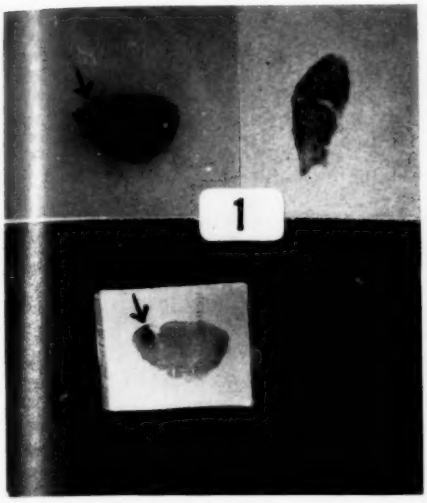
FIGURE 2.—Autoradiograms of equatorial and longitudinal sections of very small tumor after 24 hours' uptake. No necrosis.

FIGURE 3.—Autoradiograms of equatorial (*bottom*) and longitudinal sections of large tumor after 72 hours' uptake. Viable tissue is thin shell which surrounds central necrotic mass.

FIGURE 4.—Enlarged photograph of stained section from block used for lower autoradiogram of figure 3. Illustrates intercalation of necrotic and viable tissue at the boundary. Photographed through Wratten F (*red*) filter to maximize contrast between necrotic (*light*) and viable (*dark*) tissue. × 4

FIGURE 5.—Small tumor after 11 days' uptake during which tumor size increased *ca.* 7×. Highly labeled material present within necrotic region.

FIGURE 6.—Photograph of stained section from tissue block used for figure 5, showing location of necrotic zone. Wratten F filter.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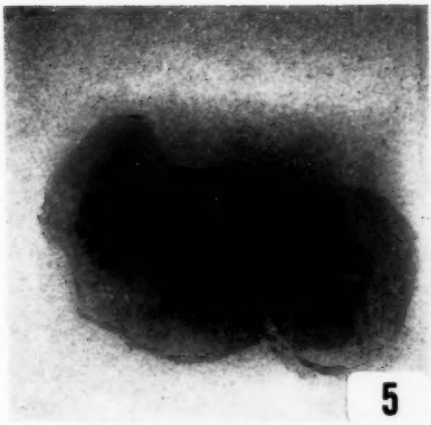
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PLATE 78

Walker tumor.

FIGURE 7.—Medium-sized tumor after 7 hours' uptake of lysine. Pattern is same as that of glycine (*cf.* fig. 3).

FIGURE 8.—Very small tumor allowed to take up lysine for 12 days. Pattern is same as that of glycine (*cf.* smaller nodule of fig. 5).

FIGURE 9.—Medium-sized tumor after 5 days' uptake of lysine. Some fragments missing from central area. *Note* inner band of high activity.

FIGURE 10.—Very small tumor after 12 days' uptake of lysine. Uniform distribution.

FIGURE 11.—Medium-sized tumor after 5 days' uptake of glycine. Some fragments missing from center. Inner band more clearly defined than in figure 9.

FIGURE 12.—Very small tumor after 12 days' uptake of glycine. Uniform distribution.

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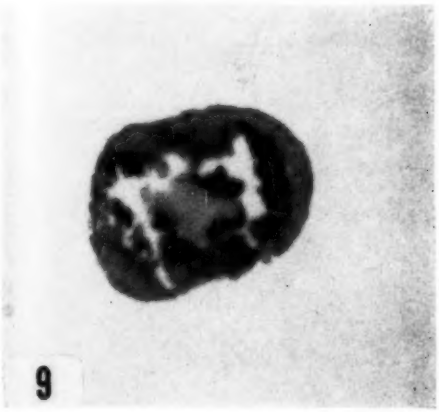
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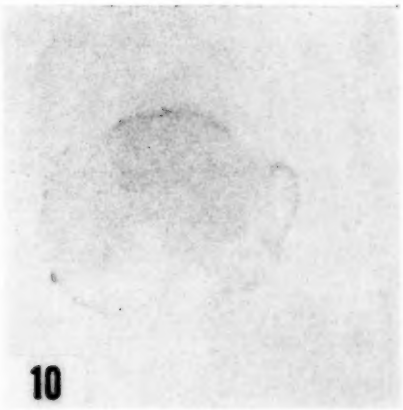
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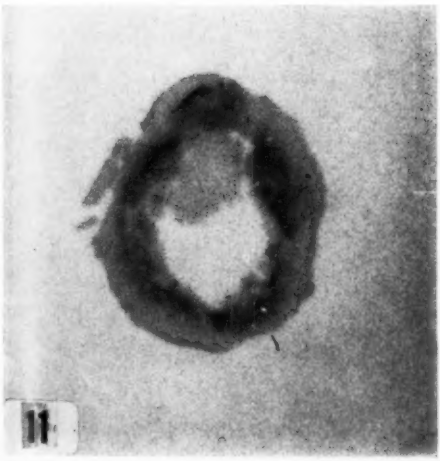
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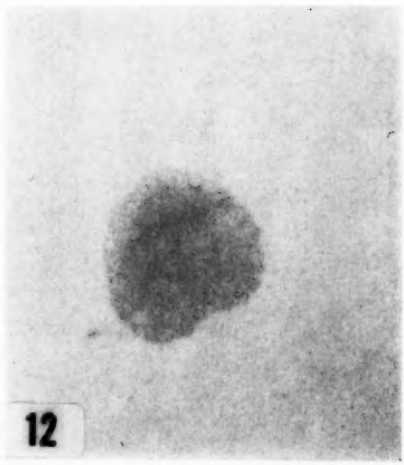
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PLATE 79

ACMCA2 fibrosarcoma.

FIGURE 13.—Very small tumor 14 days after administration of glycine. Essentially uniform distribution. Outline of small amount of necrotic tissue is visible as triangular pattern of slightly enhanced specific activity.

FIGURE 14.—Experiment 13 repeated with lysine. Crescent-shaped area of necrotic tissue occurs in center. Clear space is caused by loss of tissue fragment.

FIGURE 15.—Medium-sized tumor 7 hours after glycine administration.

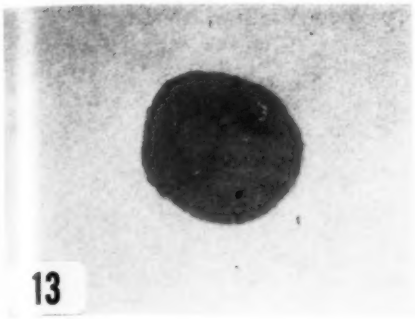
FIGURE 16.—Same at 24 hours.

FIGURE 17.—Same at 13 days. Comparatively highly labeled necrotic tissue visible in central area.

FIGURE 18.—Experiment 15 repeated with lysine.

FIGURE 19.—Experiment 16 repeated with lysine.

FIGURE 20.—Experiment 17 repeated with lysine.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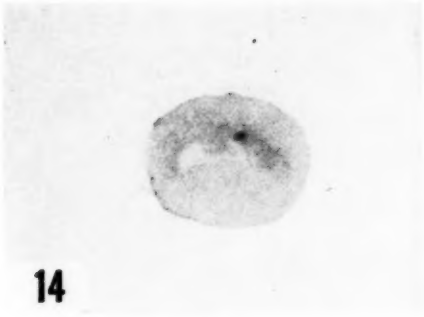
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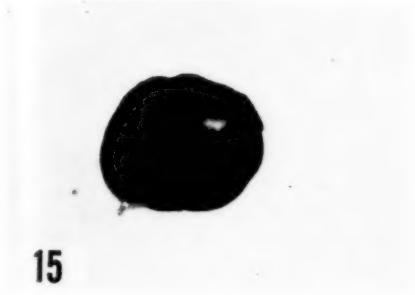
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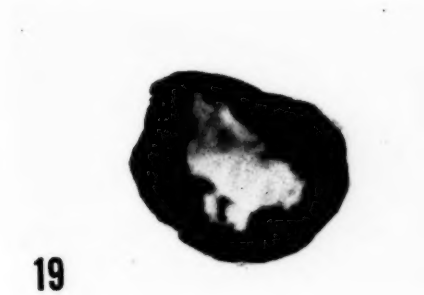
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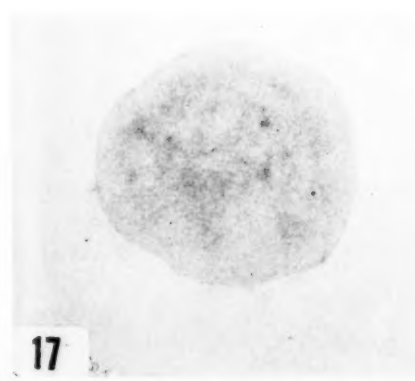
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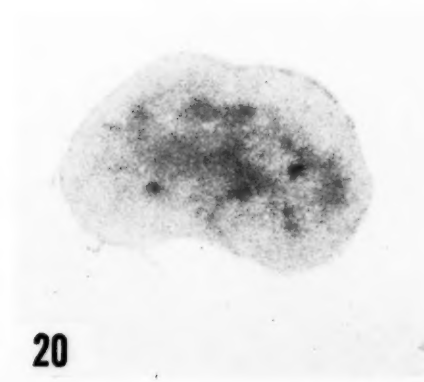
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Plate 80

S91 melanoma.

Figure 21.—Very small tumor 14 days after glycine administration. Specific activity of tumor (*inner zone*) is less than that of surrounding muscle.

Figure 22.—Experiment 21 repeated with lysine. Mouse died on 6th day.

Figure 23.—Medium-sized tumor 7 hours after glycine administration.

Figure 24.—Same at 25 hours. Tumor still highly labeled.

Figure 25.—Same at 10 days. Tumor and muscle specific activities about equal.

Figure 26.—Medium-sized tumor 7 hours after lysine administration.

Figure 27.—Same at 25 hours.

Figure 28.—Same at 10 days.

Figure 29.—Enlarged photograph of stained section of tumor used in experiment 27 illustrating open texture of tumor. Photographed through Wratten F (*red*) filter. × 4

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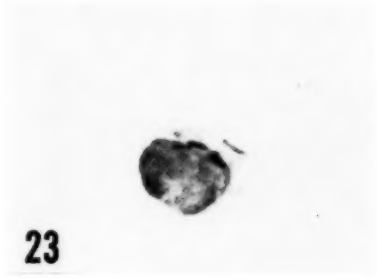
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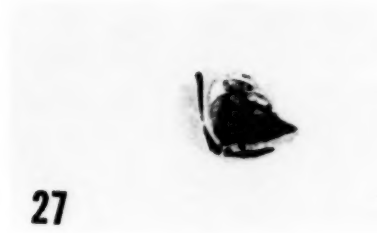
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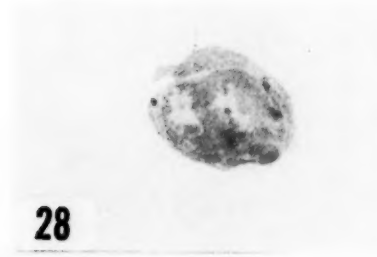
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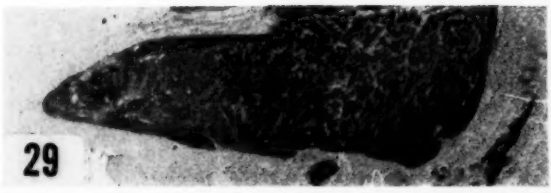
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PLATE 81

C3HBA mammary adenocarcinoma.

FIGURE 30.—Young tumor 7 hours after glycine administration. Tumor is very diffuse but vigorous uptake is apparent.

FIGURE 31.—Same at 25 hours.

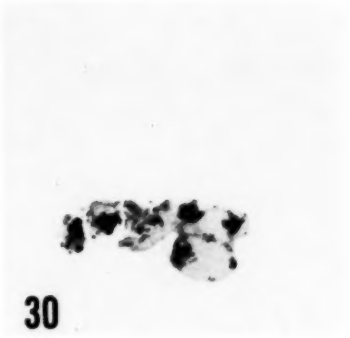
FIGURE 32.—Same at 6 days. Highly labeled material in *center* is necrotic.

FIGURE 33.—Same at 10 days.

FIGURE 34.—Young tumor 7 hours after lysine administration.

FIGURE 35.—Same at 25 hours.

FIGURE 36.—Same at 10 days. Central region contains necrotic material more highly labeled than viable portion of tumor.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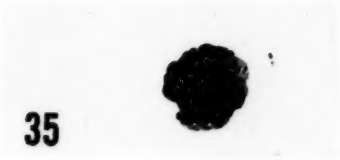
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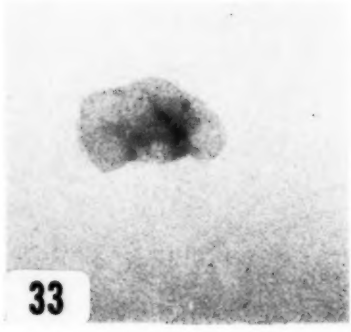
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36

Minimum Standards for the Commercial Production of Randombred and Inbred Laboratory Rats [1,2,3,4]

Summary

With the rapid progress in biological and medical research during the past decade, the problem of an adequate supply of laboratory animals has become more acute. Periodically, a shortage occurs, and the quality of animals available frequently leaves much to be desired. The Institute of Laboratory Animal Resources (ILAR), National Research Council, has been cognizant of these problems since its inception in 1952, and has constantly worked toward improvement. In considering the over-all problems, the ILAR came to the conclusion that standard procedures for the production of laboratory animals were essential to provide supplies of uniform material for the research and testing laboratories. With the beginning of the national cancer chemotherapy program, this need for uniformity was even more apparent. Consequently, the Institute of Laboratory Animal Resources, under contract with the Cancer Chemotherapy National Service Center, undertook to write minimum standards for the production of certain laboratory animals. The first of these efforts resulted in "Minimum Standards for the Production of Randombred and Inbred Laboratory Mice" (J. Nat. Cancer Inst. 19: 1137–1143, 1957). This second set of standards is concerned with rats.—**J. Nat. Cancer Inst. 22: 869–876, 1959.**

Purpose and Aim of the Minimum Standards for Rat Production

The laboratory rat is the animal of choice in a vast number and variety of studies in medical, biological, and psychological research. It is also widely utilized in testing innumerable drugs and biological products for their usefulness in alleviating human suffering. Biological standards of some drugs used in medicine require that rats be employed.

[1] Received for publication December 28, 1958.

[2] Prepared by the Committee on Standards of the Institute of Laboratory Animal Resources, National Academy of Sciences, 2101 Constitution Ave., N.W., Washington 25, D.C., for the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U.S. Department of Health, Education, and Welfare, Bethesda 14, Md.

[3] Members of the Committee: T. C. Byerly, *Chairman*; T. Aarons; R. H. Barnes; N. R. Brewer; L. R. Christensen; C. N. W. Cumming; R. J. Flynn; H. L. Foster; G. E. Jay, Jr.; J. E. Williams; J. W. Wilson.

[4] Copies of the "Minimum Standards" may be obtained from the Institute of Laboratory Animal Resources or from the Cancer Chemotherapy National Service Center.

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In research it is of great importance that the animals be as uniform and standardized as possible, in order that the results obtained in one laboratory may be repeated in a different laboratory. Uniformity also increases the accuracy of the results obtained. In addition, it speeds research by decreasing the number of repetitive experiments needed to ascertain that a particular result is not due merely to chance.

Like the standards for mice, these standards are based on the best practical and theoretical breeding and rearing knowledge now available. Since they are minimum in scope, they will be subject to revisions. However, they are workable, and if followed properly, a high quality laboratory rat will result.

Two types of uniformity must be considered in the case of the laboratory rat: genetic uniformity and environmental uniformity.

Genetic uniformity may be considered from two aspects, depending on the mating system employed. In the case of the genetically undefined rat, commonly produced commercially, consistent variability can be achieved by maintaining all commercial colonies under a system of rigidly randomized matings. This will insure that all lots of rats obtainable commercially will be uniformly variable in their genetic constitution. Experiments utilizing these rats could then be designed on the basis of their uniform genetic variability, eliminating the present source of error due to uncertainty of the degree of genetic variability of different lots of rats. It cannot be too strongly emphasized that the uniform genetic *variability* of randombred rats is just as important as the uniform genetic *invariability* of inbred strains of rats.

The genetic constancy of inbred strains of rats can only be maintained by continuing brother $\times$ sister matings, beyond the twentieth generation of such matings, for the entire life of the strain. All animals within a strain should be as closely related as possible, tracing back to only a few ancestors in every generation. The usefulness of different inbred rat strains arises from the specifically defined physiological characteristics of each strain. Thus, for example, a specific transplantable tumor will grow only in rats belonging to a specific inbred strain. Rats of different inbred strains may have different susceptibilities to certain infectious diseases or to cancer. The probability that all rats of a specific inbred strain will show the same response to a specific experimental treatment is very high, certainly far higher than that for randombred rats under similar treatment. Thus, by utilizing the very low degree of genetic variability of inbred rats, experiments can be designed in which almost all variability in the results can be attributed to the experimental treatment and other environmental conditions.

Environmental uniformity, equally as important as genetic uniformity, is in practice more difficult to attain. Since laboratory rats are produced in different localities by different breeders and under different conditions, it is essentially impossible to produce all rats under environmental uniformity. However, it is both possible and practical to approach this ideal condition. This can be accomplished, for both randombred and inbred

rats, by maintaining the animal colony under optimal conditions of cleanliness, nutrition, temperature, humidity and ventilation, and prevention of disease. Through more efficient practices in animal care, a higher level of production can be achieved, with a reduction in the number of man hours and cost of production. Although definitive experimental studies on the best cage design, nutrition, and other problems of colony management are urgently needed, a beginning toward the establishment of optimal environmental conditions in all commercial laboratory rat colonies can be made by utilizing the past experience of academic and commercial rat producers. It is out of this experience that the minimum standards presented herewith have been evolved. It should be noted that these are *minimum* standards. It is assumed that all producers will want to establish practices in their colonies which will enable them to raise rats under optimal conditions considerably surpassing those outlined in the standards.

As experience is gained in the use of these standards, changes will have to be made. As the standards are presented now, they are not intended to prevent any producer from trying out or adopting practices not specifically mentioned in the standards, if these practices are designed to improve the conditions under which the rats are raised.

The sole purpose of these standards is to aid the commercial rat breeder in the production of more uniformly healthy, well-fed rats of known genetic background and variability. If followed completely, the standards should help to provide rats with increased productivity, increased livability, and increased salability. Such rats will aid immeasurably in increasing the reliability of experimental results obtained in research in the life sciences and will thus be of great benefit to mankind.

To the User of Laboratory Rats

The rats produced under the conditions required by these standards are of superior quality. They will give more dependably uniform experimental results and more uniform bioassay results than rats produced under substandard conditions. This superior quality can be maintained only by employing breeding and husbandry practices which are more expensive than those previously used. It is therefore impossible for a rat producer meeting these standards to compete *on a price per rat* basis with dealers and breeders producing rats of average or inferior quality. The relatively small difference in price will be made up in many ways: *a*) fewer rats per experiment; *b*) fewer repeats of experiments; *c*) fewer results with questionable meaning; *d*) more uniformly repeatable results. Since the rats in any experiment are a minor cost item, it will behoove every investigator to specify to his purchasing agent that rats are to be obtained only from producers utilizing the rat standards.

Facilities and Sanitation

Facilities, equipment, and sanitary practices shall be designed to provide minimal opportunity for transmission of infectious and parasitic diseases from rats in one cage to rats in other cages, and to rats which may

subsequently occupy the same cage. Such practices will minimize the opportunity for the introduction and transmission of infectious and parasitic diseases into and within the producers' premises and from the producers' premises to the users' laboratories.

Sanitization: To make physically clean and to remove and/or destroy agencies injurious to the health of laboratory animals.

Sterilization: The act or process of killing all living cells, especially microorganisms.

Caging and equipment:

1) Cages and racks shall be made of a smooth, corrosion-resistant, impervious, easily cleaned and sanitized material, *e.g.*, stainless steel, plastic, or glass, and shall be so constructed as to eliminate or minimize cracks and crevices.

2) Minimum cage area, per nursing female (including litter), shall be 130 square inches for litters of an average size, 1 to 9 young (when the weaning age is 20–22 days).

Minimum cage area, per rat, up to 75 grams, shall be 15 square inches.

Minimum cage area, per rat, 300 grams or more, shall be 50 square inches.

Cage space shall be adjusted for rats of intermediate weights.

3) Water bottles or other watering devices, equivalent in protection of water against contamination, shall be provided.

4) Feed hoppers constructed of corrosion-resistant material shall be suspended in each cage. All hoppers, if contained within the cage, shall be covered or shall be so constructed as to prevent entry of, or gross contamination by, rats. They shall extend low enough to be reached by preweanling rats.

Bedding:

1) Bedding shall consist of sawdust, shavings, or other material not readily eaten. Hay, straw, shredded sugar cane, or any other edible material shall not be used.

2) Bedding shall be protected from exposure to vermin prior to use. It shall not be obtained from any unprotected storage source.

3) Bedding shall be dry when received and when used.

4) Storage at the rat breeder's establishment shall be under clean, dry, vermin-free conditions.

5) Bedding in mating and holding cages shall be changed weekly or oftener.

Buildings:

1) Animal room temperature shall be maintained between 70° F. and 80° F. In the summer, buildings can be cooled by painting the roofs with aluminum paint, attaching sprinkler hose to roof, and using ventilating fans inside the building. Air conditioning is recommended.

2) Adequate, draft-free ventilation shall be provided.

3) Buildings shall be rodent-proof and vermin-free. Dogs, cats, and other pets shall not be permitted in or around the colony.

4) Windows shall be adequately screened when opened for ventilation.

5) It is recommended that the relative humidity of animal rooms be maintained at 40 percent or above.

6) Animal room floors, walls, and ceilings shall be made of a smooth, hard, impervious material that can be cleaned easily. New buildings shall be constructed of materials other than wood.

Cleaning:

1) Animal house floors shall be cleaned with hot water and detergent once a week. Walls and ceilings shall be cleaned frequently, on a regular schedule, preferably by cleaning with hot water and a detergent having a satisfactory sanitizing efficiency.

2) Corridors and animal room floors shall be swept daily, with the use of a sweeping compound.

3) Cages, feed hoppers, water bottles and watering tubes, or other watering devices shall be thoroughly cleaned with hot water and detergent or otherwise sanitized before new animals are placed in the cages.

a) Mechanical cage washing machines (operating at 180° F.) used with an effective detergent are recommended. If these are used, no separate sanitization of cages is necessary under normal conditions.

b) It is recommended that all mating and holding cages, watering devices, and feed hoppers be sanitized at least once each week.

4) The water bottle or other watering or feeding device removed from any cage shall be returned to the *same* cage unless it is sanitized. This is very important to minimize transmission of diseases.

5) All refuse and dead animals shall be placed in closed containers and removed from the premises daily. Incineration is recommended. There shall be frequent inspection for and removal of sick and moribund animals.

6) Refuse containers and covers shall be sanitized thoroughly after use and before being brought into the animal room.

7) Producers shall maintain a rigorous control program against vermin, rodents, and fly-breeding places.

8) Brooms, mops, dust pans, scrapers, and other service equipment shall be kept in a specified storage room.

Disease Diagnosis and Prevention

The introduction of new stock to any breeding colony must always be considered a hazard, and every breeder must use every precaution to protect his own stock. Each inbred nucleus colony shall practice the strictest sanitary and disease-prevention measures and every effort shall be made to deliver the breeding stock in an uncontaminated and a disease-free condition.

1) All breeding stock obtained from any source, including the inbred nucleus, shall be quarantined upon receipt for at least 3 weeks in a room or building isolated from the rest of the rat colony.

2) All persons entering the colony shall wash very thoroughly, wear special shoes or clean shoe covers and clean clothing or uniforms before beginning work in the animal rooms. The clothing, uniforms, shoes, and

shoe covers are to be worn only in the animal rooms and are not to be removed from the animal room until they are removed for laundering. The clothing, uniforms, and shoe covers shall be washed at least once a week or after gross soiling or contamination.

3) Personnel handling animals shall wash their hands thoroughly at frequent intervals and shall use a disinfectant detergent immediately after handling sick animals.

4) Visitors shall not be allowed in any animal room.

5) To prevent disease dissemination all rats shall be shipped and delivered in new and nonreturnable containers. These containers shall be stored, prior to use, in clean quarters separate from the animal rooms.

6) Periodic examinations of samples of rats, taken from different cages in the colony, shall be made at competent diagnostic laboratories. A minimum of two examinations a year shall be performed at least 4 months apart.

7) Returned animal shipments (for whatever reason) shall be considered contaminated and promptly destroyed.

Genetics and Recordkeeping [5]

Rats distributed by producers shall be identified with respect to ancestry and method of mating used in their production.

Except in instances in which specific genetic or health requirements necessitate the introduction of new stock, maintenance of a closed population with no introduction of outside stock by each producer generally improves the uniformity of the stock and facilitates the maintenance of health in the colony.

The keeping of production records for each mating or breeding pen is recommended. Each cage card shall include the mortality record (number of animals and dates of death).

Randombred rats are the progeny of matings, within the closed colony, of females and males generally from unrelated litters and different cages. A numerical randomization system based on cage and litter numbers shall be used.

Inbred strains of rats are the progeny of rats resulting from brother × sister matings or from younger parent × offspring matings for twenty or more generations of such matings.

1) A small number of inbred nuclei established at different geographic locations and under the constant supervision of professional mammalian geneticists shall supply all breeding stock to commercial producers of inbred rats. There must be close liaison between the supervisor of the specific inbred nucleus and the associated producers supplied with this nucleus.

a) The inbred nucleus shall be propagated only by brother × sister matings or younger parent × offspring matings.

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[5] *Basic reference:* Genetic Standards Chapter of Handbook of Laboratory Animals, Second Edition, Institute of Laboratory Animal Resources, National Research Council. In preparation.

b) Complete records shall be kept for each animal in the inbred nucleus.

c) To assure the retention of specific genetic and physiological traits by each inbred strain, adequate performance tests shall be conducted at frequent intervals in each inbred nucleus. Performance testing at the final production level shall be done if necessary.

2) Production matings.

a) Foundation stock shall be obtained from one of the producers possessing an inbred nucleus.

b) Foundation stock shall be propagated by brother × sister or younger parent × offspring matings.

c) Only one generation of random matings within an inbred strain may be used for production of inbred rats for sale or for production of F_1 hybrids.

F_1 hybrid rats are the progeny from matings of male rats from one inbred strain and female rats from a different inbred strain. Specific F_1 hybrid combinations are useful for different specific laboratory purposes and are not generally interchangeable.

Nutrition [6]

1) Fresh feed shall be used. Food shall not be stored longer than 6 weeks after pelleting and a storage time under 4 weeks is preferable in view of the nutritional loss due to aging.

2) Feed shall be stored under clean, dry conditions in covered, pest-excluding containers, in quarters where temperatures do not exceed 75° F. Nutritional loss is minimized in relatively cool storage places.

3) No feed containing unrendered meat scraps (a source of Salmonella infections) shall be used. The feed manufacturer shall accept and use only rendered meat scraps which have been placed in clean—preferably sterile—containers (*e.g.*, five-ply kraft paper bags) immediately after rendering by the meat packer.

4) Each producer shall make sure—especially if he obtains his feed from a local mill—that the feed he uses is:

a) within normal limits of naturally occurring hormone activity and free of diethylstilbestrol contamination;

b) free of additives containing medication or antibiotics.

The results of these assays shall be clearly declared by the manufacturer.

5) If an investigator needs rats raised on a particular antibiotic-containing or other special diet, special arrangements shall be made with the rat producer. This feed, and the rats raised on it, shall be isolated from the regular feed.

6) Weaning weight (20–22 days) records and weight curves of males and females up to 12 weeks shall be available to users. The weight curves shall be redetermined at least annually, and these determinations

[6] *Basic reference:* Publication of rat nutrient requirements by the Subcommittee on Laboratory Animals, Committee on Nutrition, Agricultural Board, National Research Council. In preparation.

shall be based on a random selection of complete litters and a minimum of 50 animals of each sex in each study.

7) The rat undoubtedly is the most widely used animal species for nutrition research. Certain standards of control are of great importance in enhancing the usefulness of this species for this area of research. In particular, there is a need for standardization and avoidance of change in the formulation of the feed used by any given producer. If a change in feed formulation is of absolute necessity, the producer shall be notified immediately so that such information is available to the interested user. Furthermore, the use of "open-formula" and dated feeds is recommended.

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